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FUNCTIONS OF MACROPHAGES AND DENDRITIC CELLS IN T CELL ACTIVATION

by

© CYNTHIA JOAN GUIDOS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (IMMUNOLOGY)

EDMONTON, ALBERTA

FALL 1986

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Date 14 July 1986

Dedication

To my father, Robert Guidos, and my mother, Joan Guidos, whose love and support made it possible for me to achieve this goal.

ABSTRACT

Functional differences and cell collaboration between murine lymphoid dendritic cells (DC) and macrophages (MØ) in antigen presentation for T cell activation were analyzed with splenic DC and MØ, culture-derived bone marrow (BM)-MØ, and DC-like and MØ-like cell lines. DC were the best stimulators of allogeneic mixed leukocyte reaction (MLR), but splenic MØ and small activated BM-MØ were almost as effective. On the contrary, DC were much better than MØ at syngeneic MLR stimulation, even though the levels of surface Ia on DC, splenic MØ, and small BM-MØ, as measured by flow cytometry, were not very different. In contrast to MLR stimulation, small activated BM-MØ were the most effective antigen-presenting cells (APC) for the presentation of whole *Corynebacterium parvum* (CP) organisms, possibly by virtue of their phagocytic and lysosomal functions which could be particularly important for processing particulate antigens. Large activated BM-MØ were ineffective in stimulating MLR and CP-specific T cell proliferation. The functional differences between BM-MØ subsets could not be explained by failure to express surface Ia or to take up antigen. Nonphagocytic APC, such as DC and the DC-like line P388AD.4, had low presenting activity for CP and were much less effective at presenting glutaraldehyde-fixed CP than MØ. This suggests that DC are dependent on the shedding of soluble antigen (reduced by glutaraldehyde fixation) from the bacteria, and they may also be less efficient than MØ at processing the fixed bacteria. The Ia$^-$ MØ-like line, P388D1, was devoid of APC activity, but could greatly enhance P388AD.4-induced T cell proliferation to whole bacterial organisms. Similarly, co-culture of splenic DC and MØ produced very pronounced synergistic effects in proliferative responses to CP and keyhole limpet hemocyanin. The function of MØ in this partnership was sensitive to chloroquine and could not be replaced by MØ culture fluids or recombinant interleukin-1. Thus, MØ may contribute processed antigen in a form more suitable for presentation by DC. These results provide a rationale for the functional dichotomy between DC and MØ.

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List of Abbreviations

Immunoglobulins and chemical compunds are named in accordance with the WHO and IUPAC nomenclatures, respectively. Other abbreviations include:

A cell	accessory cell
AFC	antibody-forming cell(s)
APC	antigen-presenting cell(s)
ATCC	American type culture collection
B cell	bone marrow-derived lymphocyte
BCG	*Bacillus Calmette Guérin*
BM	bone marrow
CFA	complete Freund's adjuvant
Con A	Concanavalin A
CP	*Corynebacterium parvum*
CR	complement receptor
CSF-1	colony stimulating factor-1
CTL	cytotoxic T lymphocyte(s)
DC	dendritic cell(s)
DMEM	Dulbecco's modified minimum essential medium
DNP	dinitrophenyl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FcR	Fc receptor
FITC	fluorescein isothiocyanate
GA	poly-L-Glu:L-Ala
GLT	poly-L-Glu:L-Lys:L-Tyr

HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
I region	immune response region
Ia	I region-encoded antigen
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
I_i	invariant chain of Ia
IL-1	interleukin-1
IL-2	interleukin-2
Ir genes	immune response gene
KLH	keyhole limpet hemocyanin
L90	log 90° light scatter
LC	Langerhans cell(s)
LCM	L cell-conditioned medium
LFA	lymphocyte function-associated antigen
LN	lymph node
LPS	lipopolysaccharide
MØ	macrophage(s)
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
Mls	mixed lymphocyte-stimulating locus
MON	monomeric POL
NR	nonresponder
OPD	o-phenlyenediamine
OVA	ovalbumin
PBS	phosphate-buffered saline

PHA	phytohemaglutinin
PPD	purified protein derivative of tuberculin
PLL	poly-L-lysine
POL	polymerized flagellin
R	responder
RES	reticuloendothelial system
SAMIG	sheep anti-mouse IgG
SD	standard deviation
SN	supernatant
SRBC	sheep red blood cell(s)
T cell	thymus-derived lymphocyte(s)
TCA	trichloroacetic acid
TD	thymus-dependent
(T-G)-A-L	poly(L-Tyr:L-Glu)-poly-DL-Ala-poly-L-Lys
T_h	T helper cell(s)
TI	thymus-independent
TNP	trinitrophenyl
T_p	proliferating T cell
UV	ultraviolet

I. INTRODUCTION

The vertebrate adaptive immune response is critical for the protection of the organism against pathogens, and possibly, neoplasia. This protection is accomplished by cellular and humoral effector mechanisms, mediated by thymus-derived (T) and antibody-producing, bone marrow-derived (B) lymphocytes respectively. The activation of these cells by foreign antigens is dependent on the accessory functions of a heterogeneous third subset of cells, the most important of which are macrophages (MØ), dendritic cells (DC) and B lymphocytes. A major function of such accessory (A) cells is to "process" and "present" foreign antigens to T lymphocytes in association with A cell-surface molecules, encoded by I region genes within the major histocompatibility complex (MHC). This gene complex was originally discovered by its importance in determining tissue histocompatibility. The objective of this thesis is to establish the identity and modes of action of A cells active in the stimulation of T cell responses to various antigens. This chapter will begin with an overview of the A cell dependence of various immune responses, followed by a detailed discussion of A cell functions in lymphocyte activation and the existence of A cell heterogeneity.

A. Accessory Cell Dependence of Immune Responses

The importance of MØ to the innate immune system was recognized almost immediately upon their discovery as cells capable of engulfing particulate matter by phagocytosis (Metchnikoff, 1884). MØ and other phagocytic cells comprise a network of functionally related cells known as the reticuloendothelial system (RES) (Aschoff, 1924) which effectively removes foreign proteins, microorganisms, and particulate inorganic matter from the blood and lymph (Jaffé, 1931). The recognition that MØ and other cells have important accessory functions in specific immune responses is relatively recent. A requirement for adherent phagocytic A cells in the primary *in vitro* antibody-forming cell (AFC) response to sheep red blood cells (SRBC) was first demonstrated by Mosier in 1967 and confirmed by

Pierce (1969) and Shortman *et al* (1970).

In addition to A cells, optimal antibody formation by B cells in response to most antigens requires the cooperation of T helper lymphocytes (T_h) (Claman *et al*, 1966; Miller and Mitchell, 1967). Thus, the A cell requirement of primary *in vitro* antibody responses to such thymus- or T cell-dependent (TD) antigens may reflect the A cell dependence of antigen-induced T cell proliferation (Hersh and Harris, 1968; Oppenheim *et al*, 1968; Seeger and Oppenheim, 1970; Waldron *et al*, 1973) and lymphokine production (Nelson and Leu, 1975; Wahl *et al*, 1975), although proliferating T cells (T_p) do not always function as T_h (Ramila and Erb, 1983; Ramila *et al*, 1985). The allogeneic mixed leukocyte reaction (MLR), thought to be an *in vitro* manifestation of the allograft response, is also A cell dependent (Oppenheim *et al*, 1968), as is the activation of T cells by mitogens such as Concanavalin A (Con A) (Hedfors *et al*, 1975; Habu and Raff, 1977; Ahmann *et al*, 1978) and phytohemagglutinin (PHA) (Levis and Robbins, 1970; Lipsky *et al*, 1976; Rosenstreich *et al*, 1976; Schmidtke and Hatfield, 1976). The failure of some investigators to observe A cell dependence of mitogen-stimulated T cell activation (Hersh and Harris, 1968; Oppenheim *et al*, 1968, Waldron *et al*, 1973) was probably due to a requirement for only small numbers of A cells. Thus, stringent A cell depletion is needed to reveal A cell dependence (Rosenstreich *et al*, 1976). Moreover, A cells are inhibitory if present in excess (Diener *et al*, 1970; Folch *et al*, 1973; Rosenstreich and Mizel, 1978). T cell activation by multivalent antibodies specific for the clonotypic T cell antigen receptor or the associated monomorphic T3 complex (reviewed in Haskins *et al*, 1984; Meuer *et al*, 1984a) is also A cell dependent (van Wauve and Goossens, 1981; Kaye *et al*, 1983). In contrast, human T cells are activated in an A cell-independent manner by antibodies specific for the sheep erythrocyte receptor (T11) (Meuer *et al*, 1984b). However, the physiological significance of this activation pathway remains to be established, since no natural ligand for the T11 molecule has been described.

B cell activation by thymus-independent (TI) antigens was initially reported to be A cell independent (Diener *et al*, 1970; Shortman *et al*, 1970; Feldmann and Palmer, 1971; Feldmann, 1972a). However, later studies using more exhaustive A cell depletion procedures

demonstrated an A cell dependency for antigen-specific activation of B cells by TI antigens (Chused *et al*, 1976; Lee *et al*, 1976; Nordin, 1978; Boswell *et al*, 1980b; Martinez-Alonso *et al*, 1980; Letvin *et al*, 1981; Morrissey *et al*, 1981; Diner *et al*, 1986). Similarly, polyclonal activation by mitogens (Persson *et al*, 1977; Fernandez and Palacios, 1982; Haeffner-Cavaillon *et al*, 1982; Melchers *et al*, 1982; Bandeira *et al*, 1983; Corbel and Melchers, 1983; Fernandez and Severinson, 1983) and anti-immunoglobulin (Ig) (Mongini *et al*, 1978) were reported as A cell dependent. Although some investigators failed to observe A cell dependence of B cell activation by the mitogen lipopolysaccharide (LPS) (Ishizaka *et al*, 1977; Wong and Herscowitz, 1979; Boswell *et al*, 1980a), this was most probably due to residual MØ whose activity was enhanced by LPS. Consistent with this view, Martinez-Alonso *et al* (1980) demonstrated that LPS-induced B cell activation requires the addition of A cells only when very low LPS concentrations are used. Under some conditions, LPS may induce MØ secretion of predominantly inhibitory mediators (Kurland *et al*, 1977). This could explain the suppression by MØ of LPS-induced B cell activation seen by some investigators (Yoshinaga *et al*, 1972; Lemke *et al*, 1975). Kurland *et al* (1977) emphasized that variations in culture conditions could result in opposite conclusions with respect to the A cell dependence of mitogenic B cell stimulation. Thus, if the MØ concentration is suboptimal, additional MØ will enhance the B cell response, whereas excessive numbers are inhibitory.

Thorough evaluation of the literature indicates that activation of both T and B cells by most antigens, mitogens, and anti-receptor antibodies is A cell dependent. However, a number of heterogeneous cell types can provide accessory functions, suggesting that A cells serve distinct functions, which may vary with the activating stimulus and the responding cell.

B. Accessory Cell Functions in Immune Responses

Initially, A cells were considered to function as scavengers in antigen-specific immune responses. Burnet (1959) suggested that MØ removal of excess antigen was critical to the prevention of potentially deleterious direct lymphocyte-antigen contact. Accordingly, several early studies showed that monomeric protein antigens, less susceptible to phagocytosis than

their polymeric counterparts, are tolerogenic, whereas those antigens easily phagocytosed by MØ or removed from the circulation by the RES are immunogenic (Dresser, 1962; Frei *et al*, 1965; Golub and Weigle, 1969; reviewed in Unanue, 1972). However, since this phenomenon was observed mostly with γ-globulins known to have special tolerogenic properties, it may be unrelated to A cell function.

Feldmann and Basten proposed another model to explain MØ function in T cell-dependent B cell responses, based on their observations that T_h could collaborate with MØ and B cells across a cell-impermeable membrane by means of antigen-specific helper factors (Feldmann, 1972b; Feldmann and Basten, 1972). They suggested that MØ serve merely as a particle for presenting complexes of antigen and helper factor to B cells in a multivalent matrix, thus favoring immunity over tolerance. However, Katz and Unanue (1973) indicated that this could not explain the MØ dependence of T cell activation, nor the histocompatibility requirement for efficient T-B cooperation (Kindred and Shreffler, 1972; Katz *et al*, 1973).

The first real clues to MØ function in antigen-specific immune responses were provided by experiments demonstrating that MØ-associated antigen is usually more immunogenic *in vivo* and *in vitro* than comparable amounts of free antigen (Cline and Svett, 1968; Unanue and Askonas, 1968a; Mitchison, 1969; Spitznagel and Allison, 1970; Katz and Unanue, 1973; Pierce *et al*, 1974). Since the degradative capacity of MØ was well-established (reviewed in Unanue, 1972), it was suggested that MØ are required to degrade or process antigen into a form more suitable for interaction with immunocompetent cells (Gallily and Feldman, 1967; Feldmann and Palmer, 1971; Shortman and Palmer, 1971). A "presentation" function for MØ was proposed (Waldron *et al*, 1973) based on the importance of antigen-dependent MØ-lymphocyte clustering (Mosier, 1969; Pierce and Benacerraf, 1969; Werdelin *et al*, 1974; Lipsky and Rosenthal, 1975b) and observations that MØ could be "pre-pulsed" with antigens (Cline and Svett, 1968; Seeger and Oppenheim, 1970; Schecter and McFarland, 1970). However, the significance of antigen processing and presentation was not appreciated until the discovery that antigen-dependent MØ-T_p interactions and cytotoxic lymphocyte (CTL)-target interactions require MHC compatibility between the two interacting cell types, a phenomenon known as

MHC-restriction (Rosenthal and Shevach, 1973; Zinkernagel and Doherty, 1974). Several groups have also described a MØ secretory product which enhances T cell activation by mitogens (Gery *et al*, 1972), or antigens (Mizel and Ben-Zvi, 1980) as well as B cell activation by TD antigens (Hoffmann and Dutton, 1971; Wood and Gaul, 1974; Calderon *et al*, 1975; Hoffmann *et al*, 1979; Leibson *et al*, 1982) and TI antigens or mitogens (Rosenberg and Lipsky, 1981; Corbel and Melchers, 1983; Falkoff *et al*, 1983; Howard *et al*, 1983). Although the lymphokine mediating these functions was given different names in each of the various systems, all of these functions are now attributed to a single A cell-derived lymphokine, known as interleukin-1 (IL-1) (reviewed in Dinarello, 1984; Durum *et al*, 1985; Oppenheim *et al*, 1986).

These A cell functions will now be discussed in detail.

Class II MHC-Restricted A Cell – T Cell Interactions

Discovery of the I Region and I Region-Encoded Antigens

The immune response (I) region of the MHC was discovered as a consequence of studies on the genetic control of immune responsiveness to certain synthetic antigens (reviewed in Benacerraf, 1981). Responsiveness to hapten conjugates of poly-L-lysine (PLL) was first shown to be controlled by a single autosomal dominant "immune response" (*Ir*) gene by Benacerraf and co-workers in outbred guinea pigs (Levine *et al*, 1963). This observation was extended to inbred mice by McDevitt and co-workers who also showed that *Ir* genes are genetically linked to the MHC (McDevitt and Sela, 1965; McDevitt and Tyan, 1968; McDevitt and Chinitz, 1969). They subsequently defined the I region of the murine MHC based on genetic mapping studies of the *Ir* gene that controls immune responsiveness to the synthetic amino acid copolymer poly(L-Tyr:L-Glu)-poly-DL-Ala-poly-L-Lys ((T,G)-A-L) (McDevitt *et al*, 1972). With the aid of recombinant congenic resistant strains of mice, the I region was divided into subregions, but the only ones relevant to Ia are the A and E subregions (reviewed in Benacerraf, 1981). An I region has also been defined in several species of birds and mammals, including humans (reviewed in Götze, 1977). Highly polymorphic I

region-encoded antigens (Ia), composed of α-β heterodimers, were later identified on the surface of lymphoid cells (predominantly B cells and A cells) by mixed lymphocyte typing (Bach *et al*, 1972) and serology (David *et al*, 1973; Hauptfield *et al*, 1973).

Ia-Restricted Antigen Presentation

The importance of Ia in antigen presentation was first revealed by the studies of Shevach and Rosenthal on *Ir* gene control of responsiveness to synthetic antigens in inbred guinea pigs differing only at the I region of the MHC. Shevach *et al* (1972) observed that anti-Ia antibodies inhibit MØ-dependent, antigen-specific *in vitro* proliferation of primed T cells to both *Ir* gene-controlled and other antigens. Thus, MØ-T_p interactions appear to be dependent upon the accessibility at the MØ or T cell surface of Ia. Experiments demonstrating that efficient presentation of MØ-associated antigens requires MHC identity between MØ and T_p (Rosenthal and Shevach, 1973) provided further support for this notion. The existence of MHC- or, more specifically, I region-restriction between MØ and antigen-specific T_p was verified in mice (reviewed in Rosenthal, 1978; Schwartz *et al*, 1978) and humans (Bergholz and Thorsby, 1979; reviewed in Dausset, 1981). Most T cells specific for allogeneic class I molecules also recognize these determinants in association with self Ia (Weinberger *et al*, 1980, 1982, 1983; Rock *et al*, 1983; Golding and Singer, 1984; Ma *et al*, 1984; Singer *et al*, 1984; Mizuochi *et al*, 1986). However, some T cells can be activated by allogeneic Ia in the absence of self Ia determinants (Germain *et al*, 1982; Weinberger *et al*, 1982; Ma *et al*, 1984; Singer *et al*, 1984). The generation of T_h *in vitro* (Erb and Feldmann, 1975a; Kappler and Marrack, 1976; Pierce *et al*, 1976) and adoptive transfer of delayed-type hypersensitivity with sensitized T cells (Miller *et al*, 1975) also require I region compatibility of MØ and T cells, whereas effector CTL–target interactions are restricted in most cases by class I MHC antigens (Zinkernagel and Doherty, 1974). Although some T cells have been demonstrated to bind antigen in the absence of MHC antigens (Carel *et al*, 1983; Rao *et al*, 1984a,b; Siliciano *et al*, 1985), in most instances this is not sufficient to induce T cell activation.

The requisite recognition of foreign antigens in the association with self MHC molecules suggests that, unlike B cells, T cells can only recognize antigen on the surface of an antigen-presenting cell (APC) bearing the appropriate self MHC molecule. The Ia restriction specificity of T cells is not genetically pre-determined, but is imprinted on the T cell during intrathymic maturation (reviewed in Bevan and Fink, 1978; Zinkernagel, 1978), possibly as a consequence of interactions with Ia$^+$ cells within the thymus. Although Longo, Schwartz, and co-workers have evidence that these cells are bone marrow-derived APC (Longo and Schwartz, 1980; Longo and Davis, 1983; Longo *et al*, 1985), Lo and Sprent (1986) recently published an elegant piece of work in support of non-bone marrow-derived Ia$^+$ thymic epithelial cells.

Two hypotheses have been advanced to account for the dual specificity of T cell antigen recognition. The "two-receptor" model proposes that T cell recognition of antigen and Ia is accomplished by distinct cell surface receptors (reviewed in Zinkernagel, 1978). The "altered self" or "one-receptor" model asserts that the association of antigen and MHC on the APC surface creates a "neo-antigenic" determinant which is recognized as foreign by a single T cell receptor (reviewed in Bevan and Fink, 1978). Although many recent studies (Kappler *et al*, 1981; Matzinger, 1981; Hünig and Bevan, 1982) are inconsistent with the original version of the two-receptor model, independent recognition of antigen and MHC by distinct combining sites of a single receptor cannot be excluded. Contrary to expectations, the biochemical and molecular genetic characterization of the T cell receptor as a disulphide-linked α-β heterodimer encoded by immunoglobulin-like genes (reviewed in Haskins *et al*, 1984; Meuer *et al*, 1984a; Davis, 1985; Hood *et al*, 1985) has not provided the resolution to the one-receptor – two-receptor controversy. Possibly, more definitive answers will be provided by the use of molecular genetic technology to exchange α and β chain genes between receptors of known antigen plus MHC specificity, followed by DNA-mediated gene transfer into appropriate cells for assessment of the resulting recognition specificity. This approach is technically feasible, as shown recently by Dembić *et al* (1986), who used it to formally demonstrate that the α and β genes presumed to encode the T cell receptor can

transfer antigen plus MHC specificity from one CTL clone to another.

Relationship Between *Ir* Genes and Ia

Several lines of evidence have suggested a close functional association between *Ir* genes and Ia: 1) Both the complementing α and β *Ir* genes controlling immune responsiveness to some antigens (e.g., the synthetic random amino acid copolymer poly-L-Glu:L-Lys:L-Phe (Dorf and Benacerraf, 1975), lactate dehydrogenase$_B$ (Melchers and Rajewsky, 1975), and cytochrome *c* (Solinger *et al*, 1979)), and the structural α and β chain genes encoding an Ia molecule were mapped to the I-E and I-A subregions, respectively (Jones *et al*, 1978). Furthermore, the T_p response to these antigens requires that both *Ir* genes be expressed in the same APC (Schwartz *et al*, 1979), and is inhibited by monoclonal anti-Ia antibody (Baxevanis *et al*, 1980; Lerner *et al*, 1980). These results suggested that the genetic complementation between two *Ir* genes reflects molecular complementation between two polypeptide chains to form an Ia molecule. 2) Mutation of only three nucleotides in the structural gene encoding one polypeptide chain of an Ia molecule (McIntyre and Seidman, 1984) alters immune responsiveness to several antigens (Krco *et al*, 1981; Lin *et al*, 1981; Michaelaedes *et al*, 1981). Based on this evidence, most immunologists accepted that *Ir* genes encode Ia molecules, although formal proof has only recently been provided by the demonstration that a particular immune response defect could be corrected by making the defective mouse strains transgenic for an α chain gene (Le Meur *et al*, 1985; Yamamura *et al*, 1985).

Models of *Ir* Gene-Controlled Nonresponsiveness

The simplest explanation for *Ir* gene-controlled nonresponsiveness is that *Ir* genes encode T cell recognition structures (Benacerraf and McDevitt, 1972; Shevach *et al*, 1972). It was soon suggested, however, that *Ir* genes are expressed in APC rather than T cells, since APC from nonresponders (NR) fail to present antigen to T cells from (R x NR)F_1 hybrids (Shevach and Rosenthal, 1973). Although in some cases NR APC were shown to be defective in antigen handling (Adorini and Doria, 1981), *Ir* gene-controlled nonresponsiveness was usually attributed to defective antigen-Ia association on the surface of the APC (reviewed in

Benacerraf, 1981; Unanue, 1984; Schwartz, 1985), although there were exceptions (Kapp *et al*, 1973). Rosenthal *et al* (1977) and Benacerraf (1978), further proposed that Ia molecules actually select the antigenic determinant to be presented to T cells, since the response of (R x NR)F_1 T cells to different determinants on the insulin molecule is determined by the *Ir* genes expressed in the APC (Barcinski and Rosenthal, 1977).

The validity of the determinant selection theory was seriously challenged by demonstrations that NR T cells behave as responders if they develop in an (R x NR)F_1 environment (von Boehmer *et al*, 1978; Longo and Schwartz, 1981) and that NR APC can present a synthetic antigen of restricted heterogeneity to *allogeneic* NR or R T cells (Ishii *et al*, 1981,1982). A later study (Ishii *et al*, 1983) further concluded that, contrary to the predictions of determinant selection, the identical epitopes could be presented by R and NR APC to allogeneic T cells. Although these studies were conducted with T cells depleted of alloreactivity by the bromodeoxyuridine and light "suicide" technique, they have been criticized with respect to the possible existence of allogeneic effects. Such a criticism does not apply to the observation that NR APC can present antigen to R T cell clones (Clark and Shevach, 1982; Dos Reis and Shevach, 1983). Also inconsistent with determinant selection, *Ir* gene-controlled nonresponsiveness to several other antigens, such as (T,G)-A-L (Kimoto *et al*, 1981), myoglobin (Infante *et al*, 1981), tuna cytochrome *c* (Hedrick *et al*, 1982), and angiotensin (Thomas and Hoffman, 1982) could not be attributed to a defect in NR APC. Collectively, these observations strengthened "hole in the repertoire" models of *Ir* gene-controlled nonresponsiveness. A defective T cell repertoire could be the result of a failure to generate a particular antigen and MHC specificity by MHC-based positive selection within the thymus (Jerne, 1971; Langman, 1977; von Boehmer *et al*, 1978). Alternatively, if T cells specific for a particular foreign antigen and self MHC cross-reactively recognize a self antigen plus self MHC, they may be deleted as a result of the requirement for self-tolerance (Schwartz, 1978). In both cases, the gaps in the T cell repertoire should exist for a particular antigen only in association with self MHC. Thus, this model is consistant with demonstrations that NR APC can present antigen to allogeneic (NR or R), but not syngeneic T cells.

Biochemistry and Molecular Genetics of Ia Molecules

Biochemical analysis of murine Ia glycoproteins by immunoprecipitation and two-dimensional gel electrophoresis has identified two different murine Ia molecules (A and E), each composed of an α chain (molecular weight (MW) 34,000) and a β chain (MW 28,000) which associate noncovalently to form a heterodimer (Jones, 1977). The A_α, A_β, and E_β chains are encoded by genes in the I-A subregion, whereas the E_α chain is encoded by a gene in the I-E subregion. At least 4 human Ia antigens have been identified (reviewed in Kaufman *et al*, 1984). Protein and nucleotide sequence homologies suggest that the human leukocyte antigens (HLA) DQ (formerly called DC) and DR are the human analogs of the murine I-A and I-E molecules respectively. HLA-BR may be an additional I-E-like human Ia molecule. A fourth human Ia molecule, HLA-DP (formerly known as SB), which does not have a murine counterpart, has been identified. Both human and murine anti-Ia antibodies co-precipitate a third nonpolymorphic or invariant chain, called I_i or γ (Jones *et al*, 1979; Shackelford and Strominger, 1980). It is associated with biosynthetically immature Ia molecules in the cytoplasm, but is expressed independently of Ia at the cell surface (Sung and Jones, 1981; Koch *et al*, 1982). Since I_i associates with α and β chains in the endoplasmic reticulum, it was suggested that it facilitates intracellular transport of Ia molecules, and may be required to direct them to the cell surface (Kvist *et al*, 1982). However, the murine L cell line, a fibroblastic tumor which does not express its own Ia genes, can express transfected human or murine Ia genes in the apparent absence of endogenous I_i chains (Malissen *et al*, 1983; Rabourdin-Combe and Mach, 1983). Although the transfected genes are not efficiently expressed, the efficiency is not increased by co-transfection of the I_i gene (Malissen *et al*, 1983). Thus, the function of the invariant chain remains obscure.

Both α and β Ia polypeptides contain a hydrophobic leader sequence (absent from the mature protein), two external domains (approximately 90 residues each), a hydrophobic transmembrane domain, and a short cytoplasmic tail (reviewed in Kaufman *et al*, 1984; Mengle-Gaw and McDevitt, 1985). The membrane-distal (α_1) domain of the α chain is the only external domain lacking intrachain disulphide bonds. The domains are encoded by

separate exons, except for the transmembrane and cytoplasmic regions of the α genes, which are encoded by a single exon. Thus, the genetic organization and domain structure of class II MHC genes is very similar to that of class I MHC and immunoglobulin genes (reviewed in Hood *et al*, 1983). The strong homology of the membrane-proximal (α_2 and β_2) domains with Ig constant regions (Kaufman and Strominger, 1982; Larhammer *et al*, 1982) justifies the inclusion of class II MHC genes in the Ig gene superfamily.

Molecular genetic analysis of the location of polymorphic regions in α and β polypeptides (reviewed in Mengle-Gaw and McDevitt, 1985) has confirmed and extended the conclusions previously derived from biochemical and serological data. In general, the β chains are more polymorphic than the α chains. The two E_α alleles that have been sequenced show very little variation. Allelic variability in the A_α, A_β, and E_β molecules is clustered in 3-4 "hypervariable" segments in the membrane-distal α_1 and β_1 domains. Mengle-Gaw and McDevitt (1983) have proposed that these hypervariable regions may be juxtaposed at the surface of the Ia molecule and could thus be important for interactions with antigen or the T cell receptor. Recent evidence suggests that gene conversion may play a role in generating the extreme polymorphism of Ia molecules (McIntyre and Seidman, 1984; Mengle-Gaw *et al*, 1984; Widera and Flavell, 1984).

Both the murine and human class II genetic regions of the MHC are considerably complex. The murine I-A subregion encodes one A_α and one E_β gene, as well as three A_β genes, but it is not known if all three are functional. The I-E subregion encodes a functional E_α gene and two E_β genes, which may be pseudogenes. The existence of multiple β chain genes makes it possible that there are additional Ia molecules which have not yet been identified. At least five α chain genes and possibly as many as thirteen β chain genes are encoded by the human I region. Since one α chain can associate with β chains encoded by several different loci (Andrews *et al*, 1982; Shackelford *et al*, 1983), there is the potential for many more human Ia molecules than have so far been described.

Individuals heterozygous for any of the genes encoding α or β Ia polypeptides have the potential to express a greater variety of Ia molecules than homozygotes, since α and β chains of

different haplotypes can cross-associate to form "hybrid" F_1 Ia molecules with unique determinants recognizable by alloreactive (Fathman and Hengartner, 1978) or antigen-reactive, Ia-restricted (Kimoto and Fathman, 1980) T cells. Thus, a mouse heterozygous for both the I-A and I-E subregions would express 8 different Ia molecules, assuming that there is no preferential association between α and β chains of the same haplotype. Kimoto and Fathman (1980) suggested that such cross-association of α and β chains would provide heterozygotes with a larger repertoire of Ia-restricted T cells than homozygotes, thus reducing the likelihood of being a NR to a particular antigen. However, more recent data show that the association of α and β chains to form Ia molecules is not random. McNicholas *et al* (1982) and Conrad *et al* (1982) reported a quantitative deficiency of hybrid I-E molecules on spleen cells of certain F_1 strain combinations due to preferential association of the E_α and E_β chains of the parental haplotypes. Glimcher *et al* (1983a) generated an I-A mutant subline from a murine H-$2^{k,d}$ B cell-B lymphoma hybridoma that was later shown not to express significant amounts of hybrid I-A molecules (Schlauder *et al*, 1985). Germain *et al* (1985b) transfected murine A_α and A_β gene pairs into non-I-A-expressing mouse L cells and observed significant surface I-A expression only when the co-transfected α and β genes were from the same haplotype. These results indicate that the functional association of α and β polypeptides for a particular antigen is probably not random, and may actually be impossible in some instances. Germain *et al* (1985b) suggested that such preferential association of certain A_α and A_β chains may provide the selective pressure to maintain the strong linkage disequilibrium which exists between some A_α and A_β genes.

Antigen-Ia Interactions

Despite the conclusions of numerous studies that *Ir* gene-controlled nonresponsiveness could not be explained solely as a selective failure of NR Ia to interact with a particular antigen, there is evidence that antigen-Ia interactions are functionally relevant in T cell activation. Schwartz and colleagues have championed this view (reviewed in Schwartz, 1985) based on their analysis of the antigen and MHC fine specificity of cytochrome *c*-specific T cell clones and hybridomas. Initial studies demonstrated that two complementing *Ir* genes, which

map to the I-A and I-E subregions of the MHC, control the T cell response to the immunodominant fragment (residues 81-104) of pigeon cytochrome *c*. The fine antigen specificity of some T cell clones and hybridomas (determined by patterns of cross-reactivity with cytochromes *c* from other species or various chemical derivatives thereof) was dependent on the Ia haplotype of the APC, suggesting that antigen and Ia molecules interact prior to or during T cell activation (Heber-Katz *et al*, 1982, 1983; Hedrick *et al*, 1982). A similar conclusion was inferred from more recent data showing that a change in potency of a particular antigen-Ia ligand for a T cell clone resulted from the presence of a different allelic form of the Ia molecule on the APC (Ashwell and Schwartz, 1986). This was not due to alteration of the T cell receptor's affinity for that ligand, since the point at which the dose response curves shifted with increasing numbers of T cells was identical for both antigen-Ia combinations.

Based on these observations, Heber-Katz *et al* (1983) proposed that antigen-Ia interactions only occur in the presence of the T cell receptor, as a trimolecular or ternary complex, an idea which is consistent with both determinant selection and deficient T cell repertoire models of *Ir* gene-controlled nonresponsiveness. According to this model, the Ia molecule should possess distinct sites for interaction with antigen and the T cell receptor, termed the desetope and histotope, respectively. The corresponding sites on the antigen are referred to as the agretope and epitope, respectively. An agretope has been identified on cytochrome *c* (Hansburg *et al*, 1983a) and lysozyme (Manca *et al*, 1984), and an epitope has been identified on cytochrome *c* (Hansburg *et al*, 1983b) and the synthetic amino acid polymer poly-18 (Fotedar *et al*, 1985).

The ternary complex model is supported by the recent results of Watts *et al* (1986) who used a novel technique to detect antigen-Ia interaction. It had been previously shown that purified I-A and an ovalbumin (OVA) fragment incorporated into a planar lipid membrane on a glass substrate could stimulate interleukin-2 (IL-2) release from an OVA-specific T cell hybridoma (Watts *et al*, 1984, 1985). In the most recent study, I-A^d and the OVA peptide were labeled with a red or green fluorescent dye, respectively, before incorporation into the membrane. Resonance-energy transfer from the peptide to the I-A molecule in an evanescent

wave-field could only be observed in the presence of the T cell hybridoma, suggesting that the T cell receptor stabilizes the antigen-Ia interaction.

Other lines of evidence suggest that antigen-Ia association can occur without the T cell receptor. First, nonimmunogenic antigen analogs can compete with structurally related immunogenic antigens to inhibit T cell activation (Werdelin, 1982; Rock and Benacerraf, 1983a, 1984a). Similarly, antigen-pulsed APC lose the ability to present certain allogeneic Ia determinants (Rock and Benacerraf, 1984b). The inhibition is due to competition at the level of the APC, because the effect is observed when APC, but not T cells, are pre-pulsed with the competing antigens (Werdelin, 1982; Rock and Benacerraf, 1983a). This was recently shown more definitively by pre-pulsing fixed APC (Falo *et al*, 1985; Buus and Werdelin, 1986a). Secondly, a lysozyme peptide was shown to bind purified I-A^k with a 10-fold higher association constant than it bound purified I-A^d, in accordance with the *Ir* gene phenotypes of these haplotypes (Babbitt *et al*, 1985). Thirdly, several groups have reported the production by APC of antigen-Ia complexes which can activate T cells in the absence of antigen and APC. The complexes are usually tightly (perhaps covalently) linked (Erb and Feldmann, 1975b; Erb *et al*, 1976; Puri and Lonai, 1980; Puri *et al*, 1985), but the two moieties may also be reversibly associated (Friedman *et al*, 1983).

The general applicability and significance of these findings remains to be established. Schwartz (1985) suggested that the competitive inhibition phenomenon may only be demonstrable with antigens which have an unusually high affinity for Ia, since he did not observe competition for Ia by non-cross-reactive cytochrome *c* analogs. Moreover, the highly charged nature of the copolymers used in most studies may have facilitated their interaction with Ia or the APC surface. With respect to the Babbit *et al* (1985) finding, more antigen-Ia combinations need to be tested before generalizations can be made. It is possible, however, that the antigen used in this study represents an exception to the rule, and that in most instances, the affinity of antigen-Ia association may not be sufficiently high to be detected in the absence of the T cell receptor. Finally, the existence of antigen-Ia complexes has not been generally accepted, due to lack of substantiation of these results in other systems. Based on our current

understanding of T cell activation, it is not readily apparent why a soluble antigen-Ia complex might be necessary; such a complex may even defeat the purpose for which MHC-restricted recognition of antigen may have evolved; namely, to destroy intracellular parasites. Thus, although the existence of antigen-Ia association prior to interaction with the T cell is still open to question, it is clear that antigen and Ia interact as part of a ternary complex. This interaction could reflect a receptor-like function of the Ia molecule, but Parham (1984) suggested that the interaction of antigen, Ia, and the T cell receptor can only occur if the repulsive forces between antigen and Ia are insufficient to prevent the complex from forming. Thus, he sees antigen-Ia interaction as the lack of a negative interaction, rather than a positive interaction.

T Cell Recognition Sites on Ia Molecules

Several approaches have demonstrated that different T cells can recognize multiple sites (or histotopes) on a particular Ia molecule. Early studies utilized monoclonal anti-Ia antibodies to block antigen-specific T cell proliferation and revealed that individual anti-Ia antibodies had differential blocking patterns for a number of antigen-specific T cell clones (Clark *et al*, 1982). This suggested that different T cell clones recognize antigen in association with distinct Ia histotopes. Needleman *et al* (1984), using a similar approach, have suggested the existence of multiple functional T cell recognition sites on I-E molecules. Such antibody inhibition could be due to a direct competition between antibody and the T cell receptor for the same Ia determinant, or to steric effects. A recent study by Frelinger *et al* (1984) supports the latter mechanism, since T cells restricted to combinatorial F_1 I-A determinants could nonetheless be blocked by monoclonal antibodies specific for only the A_α or A_β chain. Regardless of whether anti-Ia inhibition is due to direct blocking of T cell-Ia interaction or to indirect steric effects, the differential blocking patterns suggest that there are multiple sites of T cell interaction with Ia molecules.

An alternative approach to studying functional sites on Ia molecules is to correlate Ia sequence variation with changes in *Ir* gene function. This has been done with spontaneous and induced Ia mutants. For example, the murine *bm12* mutant, whose I-A molecule differs from

the parental B6 I-A^b molecule by only three residues (McIntyre and Seidman, 1984), was used to demonstrate that at least two functional sites (one of which was altered by the *bm12* mutation) exist on the I-A^b molecule. Thus, some alloreactive T cell clones are activated equally well by I-A^b and I-A^{bm12}, whereas others are only activated by I-A^b (Beck *et al*, 1983). Similarly Kanamori *et al* (1984) observed that some antigen-specific T cell clones discriminate between B6 and *bm12* APC, whereas others do not. Glimcher *et al* (1983c) derived a similar conclusion from studies utilizing a panel of APC lines which had been mutagenized and immunoselected for expression of mutant I-A^k molecules. Although the failure of some antigen-specific T cell hybridomas to be activated by antigen and mutant APC could have been due to ineffective interaction between antigen and mutant I-A^k molecules, this is unlikely, since some mutant I-A^k-bearing cell lines also fail to activate autoreactive T cell hybridomas (Glimcher *et al*, 1985).

All of these studies support the existence of multiple sites on Ia molecules capable of interaction with T cells, but they do not delineate the nature or the location of such sites. Genetic evidence suggests that T cells recognize predominantly Ia conformational determinants (Tse *et al*, 1985), possibly formed by the association of α and β polypeptides (Kimoto and Fathman, 1980). The availability of cloned class II MHC genes and techniques for achieving stable expression of cloned Ia genes in tranfected cell lines have allowed a more precise delineation of the sites on Ia molecules important in T cell activation. Folsom *et al* (1985) and Germain *et al* (1985a) utilized "exon-shuffled" E_β and A_β constructs to determine the contribution of the β_1 and β_2 domains of the I-A and I-E molecules to T cell recognition. Consistent with the localization of polymorphic sites to the β_1 domain by nucleotide sequencing (reviewed in Mengle-Gaw and McDevitt, 1985), these investigators found that most critical determinants are localized to the β_1 domain. Lechler *et al* (1986) confirmed this and determined that both halves of the β_1 domain contribute to the specificity of T cell recognition. Transfection of haplotype mismatched α and β gene pairs revealed that a large proportion of T cells recognize conformational determinants that depend on polymorphic regions in both the α and β chains (Lechler *et al*, 1986). However, in contrast to activation of most Ia-restricted T_p

and T_h, triggering of the lytic mechanism of some Ia-restricted CTL was shown to depend only on recognition of the $A_{\beta 1}$ domain (Golding *et al*, 1985). Overall, most class II gene transfection studies support the genetic evidence that Ia-restricted T cells recognize conformational Ia determinants formed by the association of α and β polypeptide chains.

Accessory Molecules on T Cells Which Facilitate Interaction With Ia^+ APC

Two non-overlapping subsets of murine peripheral T cells have been distinguished based on the cell-surface expression of the L3T4 and Lyt-2 differentiation antigens (reviewed in Dialynas *et al*, 1983; Swain, 1983). The corresponding human antigens are T4 and T8, respectively (reviewed in Reinherz *et al*, 1983). Most T_h and T_p are $L3T4^+$, $Lyt\text{-}2^-$, whereas most CTL are $L3T4^-$, $Lyt\text{-}2^+$, but this functional correlation is not absolute. Rather, these differentiation markers are more strongly correlated with the MHC-restriction specificity of T cells (reviewed in Dialynas *et al*, 1983; Reinherz *et al*, 1983; Swain, 1983). Thus, most $L3T4^+$, $Lyt\text{-}2^-$ T cells are class II MHC-restricted, whereas most $L3T4^-$, $Lyt\text{-}2^+$ T cells are class I MHC-restricted.

The correlation of L3T4 expression with class II MHC-restriction specificity and the observation that anti-L3T4 antibody blocks T cell activation have led to the proposal that the L3T4 molecule interacts with nonpolymorphic regions of Ia antigens (Marrack *et al*, 1983; Wilde *et al*, 1983; Swain *et al*, 1984; Gay *et al*, 1986). However, anti-L3T4 antibody inhibits T cell activation by A cells bearing hybrid class I/class II MHC antigens that lack the nonpolymorphic $I\text{-}A_{\beta 2}$ domain (Golding *et al*, 1985), suggesting that L3T4 may also interact with other strucures on A cells. This interaction is not essential, since $L3T4^-$ variants of T cell hybridomas are functional (Marrack *et al*, 1983), but it may be especially important for stabilizing T cell-APC interaction when the affinity of the T cell receptor for antigen plus Ia is low (Greenstein *et al*, 1984, 1985). L3T4 may have additional functions in T cell activation, since inhibition of T cell activation by anti-L3T4 antibody can be observed in the presence of Ia^- A cells (Bekoff *et al*, 1985; Wassmer *et al*, 1985), and is not obtained with all A cell types (DeKruyff *et al*, 1985a; Malek *et al*, 1985).

Antigen Uptake

Of fundamental importance to APC function is the ability to take up a large variety of antigens. MØ engulf particulate antigens such as microorganisms or red blood cells by phagocytosis, which can be separated into distinct attachment and ingestion phases (reviewed in Horwitz, 1982). Attachment of particles to the MØ membrane is dependent on the surface properties of both the particle and the MØ, and is greatly facilitated if the particles are coated (opsonized) with specific antibody or complement, for which the MØ has specific receptors. Attachment can also take place by mechanisms not dependent on antibody or complement. For example, lectin-like molecules on the surface of *E. coli* and *S. typhi* mediate binding to mannose residues on MØ (Bar-Shavit *et al*, 1977). Conversely, mannose receptors on MØ are involved in the binding of yeast and zymosan (Warr, 1980). Lectin-like receptors on the MØ surface and divalent cations have also been implicated in the attachment of bacteria (Freimer *et al*, 1978; Ogsmundsdóttir *et al*, 1978). The formation of antigen-independent MØ-lymphocyte clusters has been postulated to involve the same class of receptors (Weir and Ogsmundsdóttir, 1977; Lipsky and Rosenthal, 1975a). This interaction is transient, not I region-restricted, and does not lead to lymphocyte activation, but may provide a means to bring MØ and lymphocytes together to facilitate immunologically relevant interaction.

Following attachment, MØ extend pseudopods which move around the particle and fuse at the other side to enclose it in a membrane-bound vacuole or phagosome. The phagosome then fuses with cytoplasmic lysosomes. Lysosomal hydrolytic enzymes released into the phago-lysosome help to degrade the ingested particle (reviewed in Stossel, 1974). Thus, these destructive enzymes are sequestered from the rest of the cell and only released into a contained microenvironment when needed.

Soluble antigens can be ingested and degraded by a similar process, known as pinocytosis (Lewis, 1931). This has been extensively studied using horseradish peroxidase and was reported not to involve binding to the MØ surface (Steinman and Cohn, 1972). Presently, however, it is not clear whether soluble proteins can be pinocytosed without first binding to the MØ membrane, since it has more recently been shown that many soluble glycoproteins,

including horseradish peroxidase (Stahl *et al*, 1978), can bind to the MØ surface *via* carbohydrate receptors and be ingested by a process known as receptor-mediated endocytosis (Stahl *et al*, 1980). Receptor-ligand complexes accumulate in clathrin coated pits on the membrane before internalization in vesicles known as receptosomes (reviewed in Pasten and Willingham, 1981). Ultimately, the receptosome also fuses with the lysosome. MØ receptors for the Fc portion of Ig (FcR) and other specific receptors may also facilitate the interiorization of some soluble proteins. For example, the uptake of normal rabbit IgG and angiotensin by mouse MØ was shown to occur predominantly *via* FcR and angiotensin receptors, respectively (Heusser *et al*, 1977; Thomas and Hoffman, 1984). However, the angiotensin receptor on MØ probably has little to do with MØ function as APC; rather, angiotensin may regulate MØ production of angiotensin-converting enzyme, which is involved in the maintenance of blood pressure (Hinman *et al*, 1979). Lastly, some proteins may bind nonspecifically to undefined plasma membrane structures prior to ingestion by MØ (reviewed in Chestnut and Grey, 1985).

The relative importance of these different routes of antigen uptake to immune induction has not been established. For some soluble protein antigens, pinocytosis is not required, since significant binding can occur under conditions (e.g., 4° C) which inhibit the pinocytic pathway (Cohn, 1966; Waldron *et al*, 1974). Presumably, phagocytosis is the predominant mode of ingestion of particulate antigens, although it is also possible that such antigens shed membrane fragments which can be taken up *via* pinocytosis or receptor-mediated endocytosis. It is not known whether the uptake of antigens by different routes has different functional consequences.

Antigen Processing

Although it has long been known that MØ actively degrade most antigens (reviewed in Unanue, 1972), the relevance of this process to immune induction was not initially clear since in most cases, the MØ-catabolized antigens were not immunogenic (Kölsch and Mitchison, 1968; Unanue and Askonas, 1968b; reviewed in Unanue, 1972). However, it was demonstrated

that a minute proportion of the antigen taken up by MØ is retained on the plasma membrane either in its native form, or only partially degraded (Unanue *et al*, 1969; Cruchaud and Unanue, 1971), and this was thought to be the immunogenic moiety. Waldron *et al* (1974) demonstrated that a temperature-dependent lag phase is required before MØ-associated antigen becomes immunogenic, as assessed by the capacity to induce antigen-specific T cell proliferation. This suggested that MØ metabolic processes are required to render antigen immunogenic for T cells. Several earlier studies by Fishman (1959, 1961), Fishman and Adler (1963), and others (Askonas and Rhodes, 1965) had implicated the generation of a supraimmunogenic antigen-RNA complex as the outcome of the MØ antigen handling process, but this was shown to be incorrect (Roelants and Goodman, 1969).

Circumstantial evidence indicates that the MØ-associated antigen which is immunogenic for T cells has been structurally altered. For instance, antibodies specific for native protein antigens usually fail to inhibit antigen-specific T cell proliferation (Ellner and Rosenthal, 1975; Werdelin and Shevach, 1979; Farr *et al*, 1979; Chestnut *et al*, 1980). There are several possible explanations for this. First, in some cases, immunologically relevant antigenic moieties may be sequestered by the MØ in sites inaccessible to antibody (Ellner and Rosenthal, 1975; Ellner *et al*, 1977). Second, the density of antigenic determinants present on the MØ surface may be too low to allow high affinity binding by antibody. In this regard, Thomas and Shevach (1978) demonstrated that the T cell proliferative response to trinitrophenylated (TNP)-MØ could be inhibited by high titer anti-TNP antisera, but if the MØ had been first cultured at 37° C for 24 hr, the density of TNP was only 2% of that on freshly modified MØ, and antibody inhibition was no longer obtainable. More recently, the same group achieved inhibition of TNP-OVA-specific T cell proliferation with anti-TNP antibody (Shevach *et al*, 1982). The mechanism of inhibition in this case could involve capping and shedding of TNP-OVA by anti-TNP antibody, since the inhibition was only observed with divalent antibody. Clayberger *et al* (1983) similarly achieved inhibition of hapten-specific T cell proliferation by anti-hapten antibody.

A third explanation for the lack of effect of antibody to the antigen to inhibit T cell activation (in most cases) is provided by studies documenting that most B cells are unable to recognize cross-reactivity between native and denatured or fragmented forms of the same globular protein antigen (Ishizaka *et al*, 1975; Chestnut *et al*, 1980; Maizels *et al*, 1980; Thomas *et al*, 1981). T cells, in contrast, do not generally distinguish between native and denatured or fragmented forms of proteins (Gell and Benacerraf, 1959; Chestnut *et al*, 1980; Maizels *et al*, 1980; Thomas *et al*, 1981). In fact, the same T cells can respond to both forms of the antigen (Chestnut *et al*, 1980; Chestnut and Grey, 1985). Moreover, immunization with synthetic antigen fragments activates T cells capable of responding to the intact antigen, whereas antibodies elicited in this manner bind the immunizing peptide, but not the native antigen (Thomas *et al*, 1981). The difference in reactivity patterns between T and B cells were interpreted to mean that MØ catabolize antigen into small peptides that lack a stable tertiary conformation, thus destroying the conformational determinants recognized by most B cells (reviewed in Sela, 1969), but leaving sequential T cell determinants intact (Chestnut and Grey, 1985). Nevertheless, it should be possible to achieve antibody blocking of T cells clones with the same antigenic fine specificity as a monoclonal antibody. This has recently been demonstrated by Corradin and Engers (1984).

Ziegler and Unanue (1981) extensively characterized MØ handling of the bacteria *Listeria monocytogenes* and also concluded that T cells recognize catabolized or "processed" antigen. They used a short term binding assay in which T cells were specifically depleted by binding to antigen-pulsed MØ monolayers, and found that after antigen uptake, a 30-60 min incubation at 37° C is required before T cells can be retained by MØ-associated antigen. Fixation of APC abolishes their antigen-processing capacity, but if antigen-pulsed MØ are incubated for 30-60 min at 37° C before fixation, they retain the ability to bind T cells. Although the nature of the MØ antigen-handling event was not determined, a subsequent study revealed that the appearance of MØ-associated immunogencity is substantially inhibited by pretreatment of MØ with lysosomotropic agents such as chloroquine or ammonium chloride (Ziegler and Unanue, 1982). These weakly basic agents become concentrated inside the

lysosomes and decrease saltatory lysosomal movements (D'Arcy Hart *et al*, 1983) and lysosome-phagosome fusion (Gordon *et al*, 1980), and raise lysosomal pH (Okhuma and Poole, 1978). Possibly as a result of these effects, these agents greatly decrease the catabolism of *L. monocytogenes* without inhibiting antigen uptake. Together these results strongly suggest that MØ-associated antigens require degradation *via* a chloroquine- and ammonium chloride-sensitive (presumably lysosomal) pathway to be rendered immunogenic for T cells.

Lysosomes contain several types of proteases (reviewed in Barrett, 1975), any or all of which may be important for antigen processing. Buus and Werdelin (1986b) used group-specific inhibitors of different proteinases to determine which lysosomal enzymes are relevant for antigen processing by guinea pig MØ. Interestingly, they found that a group-specific inhibitor of cysteine (thiol) proteinases inhibits the degradation and presentation of dinitrophenyl (DNP)–PLL, but does not inhibit presentation of the synthetic amino acid random copolymer poly-L-Glu:L-Ala (GA). An inhibitor of aspartic (carboxyl) proteinases enhances presentation of GA. Presentation of the purified protein derivative of tuberculin (PPD), a complex mixture of peptides, is only moderately decreased by both inhibitors. It is likely that the processing of such complex antigens requires the concomitant action of several proteinases with distinct substrate specificities. A relatively simple antigen like DNP-PLL contains only one type of peptide bond, and this probably accounts for the complete inhibition of its processing by a single group-specific proteinase inhibitor. The enhancement of GA presentation by an aspartic proteinase inhibitor suggests that this proteinase destroys some epitopes of this antigen. Thus, the production of processed immunogenic moieties probably involves a balance between creation and destruction of various epitopes (and possibly agretopes) by different classes of lysosomal proteinases. This implies that the immunogenicity of a particular antigen may depend not only on the existence of immunocompetent lymphocytes capable of recognizing it, but also (perhaps even primarily) on its pattern of susceptibility to degradation by lysosomal proteinases. Thus, an immune response to some nonimmunogenic proteins might be obtainable by selective inhibition of certain lysosomal proteinases.

Several studies have attempted to determine the cellular location of the MØ-associated antigen that is immunologically relevant. The MØ-bound antigen that is ultimately recognized by T cells is assumed to be a very small proportion of that initially taken up, since the majority is extensively degraded (Calderon and Unanue, 1974). Unanue *et al* (1969) originally reported data which suggested that the immunologically relevant antigen is located on the MØ membrane and is either not internalized, or is relocated to the cell surface without being substantially degraded. Consistent with this view, trypsinization of antigen-pulsed MØ was reported to abrogate their ability to present antigen (Unanue, 1978). However, since MØ function was not MHC-restricted in this system, it is possible that the surface-associated antigen was being shed and re-presented after further processing by MØ in the responder population and thus was not directly immunogenic. Contrary to Unanue's findings, the studies of Waldron *et al* (1974), Ellner and Rosenthal (1975), and Ellner *et al* (1977) support an intracellular location for immunologically relevant antigen, since in these systems, trypsin treatment of antigen-pulsed MØ does not affect their capacity to induce T cell proliferation. Presumably, however, T cells recognize surface-associated antigen, suggesting that the sequestered immunogenic moieties must be re-expressed on the MØ surface before MØ-T cell interaction (Ellner and Rosenthal, 1975). This possibility is consistent with the demonstration that fixation of APC immediately following enzymatic removal of surface-associated antigen abolishes their immunogenicity (Chestnut *et al*, 1982a). Therefore, immunologically relevant antigen exists in an intracellular location and is relocated to the cell surface by a process requiring metabolic energy.

Recently, Shevach and co-workers presented data which they considered inconsistent with the prevailing view that an intracellular phase of antigen handling is required to render antigens immunogenic (Malek *et al*, 1983). The system involved pulsing MØ with ^{125}I-labeled poly-L-Glu:L-Lys:L-Tyr (GLT) and TNP-modifiying the cell surface at various times thereafter (Malek and Shevach, 1982). Surface-associated GLT was identified as ^{125}I-labeled material precipitable by anti-TNP antibody, whereas intracellular GLT was ^{125}I-labeled but not anti-TNP precipitable. They observed that a minute (3-6%) amount of intracellular GLT is relocated to the cell surface, but concluded that this amount is too small to be immunologically

relevant. However, in view of studies already cited showing that only a small percentage of MØ-associated antigen is immunologically relevant, this low amount of re-expressed antigen should not be discounted. It was also concluded that only surface GLT is immunologically relevant, since anti-TNP antibody could inhibit presentation of a TNP-modified protein antigen (Shevach *et al*, 1982) at a time when only surface GLT was detectable. This surface-associated GLT was assumed to be unprocessed, based on precipitability with anti-GLT antibody, but this does not prove that the GLT was unprocessed; antibodies specific for synthetic antigens which lack well-defined secondary or tertiary structures often recognize sequential determinants that may not be destroyed by processing (reviewed in Sela, 1969). Furthermore, such structurally simple antigens may not need to be processed; this is true for GLT (Falo *et al*, 1985). Based on these alternative interpretations, the results of these studies do not constitute a serious challenge to the concept that most antigens require an intracellular phase of handling by MØ or other A cells.

In an attempt to locate the immunologically relevant pool of MØ-associated antigen, Unanue's group recently analyzed the fate of ^{125}I-labeled *L. monocytogenes* organisms following ingestion by MØ (Allen and Unanue, 1984a; Allen *et al*, 1984a). This study essentially confirmed earlier results with soluble protein antigens (Unanue and Askonas, 1968b; Calderon and Unanue, 1974; Malek and Shevach, 1982). By 4 hr after uptake, most (60-70%) of the cell-bound radioactivity is released into the supernatant as acid-soluble material, 3-4% remains membrane associated in the form of small (MW 10,000) peptides, and approximately 10% is released in acid-insoluble macromolecular form (MW 14,000-150,000). Whereas chloroquine inhibits the degradation of *Listeria* to acid-soluble material by 50%, neither the release of the macromolecular peptides nor the appearance of the membrane-associated radioactivity is affected. Finally, in contrast with their earlier studies with keyhole limpet hemocyanin (KLH) but in agreement with other studies, Allen *et al* (1984a) found that trypsin treatment of antigen-pulsed MØ does not affect immunogenicity. Although the membrane-associated peptides can be re-presented by other MØ and the released macromolecular peptides are weakly immunogenic, neither of these forms represents the

immunologically relevant antigen, since their appearance is not prevented by chloroquine. Thus, although this study identified both intra- and extralysosomal pathways of antigen degradation, it failed to locate immunologically relevant antigen fragments. These could be located at some unidentified intracellular site, or on the membrane in a trypsin-inaccessible location. Regardless of their location, such fragments presumably represent a minute fraction of the antigen initially taken up by MØ and may not be detectable by conventional means.

The description by several groups of tightly linked antigen-Ia complexes released by antigen-pulsed MØ (Erb and Feldmann, 1975b; Erb *et al*, 1976; Puri and Lonai, 1980; Puri *et al*, 1985) raises the possibility that antigen handling by MØ is more complex then simple antigen fragmentation. However, several recent studies have demonstrated that glutaraldehyde-fixed (metabolically inactive) or chloroquine-treated APC can present fragments of OVA, lysozyme, myoglobin, and cytochrome *c*, but not the native antigens (Shimonkevitz *et al*, 1983, 1984; Allen *et al*, 1984b; Streicher *et al*, 1984; Kovač and Schwartz, 1985), suggesting that at least for these antigens, processing appears to be nothing more than fragmentation.

What is the basis for the antigen-processing requirement? Recent evidence suggests several possibilities. The requirement for fragmentation of some antigens suggests that there may be limits on the size of ligand (antigen plus Ia) that can be accommodated by the T cell receptor, thus necessitating a reduction in size for most antigens. The results of Lee *et al* (1982) are consistent with this possibility. They reported that chloroquine pretreatment of APC abrogates the presentation of the large (MW millions) protein antigen polymeric flagellin (POL), but not PPD (MW 2,000-6,000). Buus and Werdelin (1986c) have recently confirmed this finding.

Reduction in antigen size may not be the only relevant consideration with respect to the requirement for antigen processing. DeLisi and Berzofsky (1985) analyzed 12 antigens for which the T cell immunodominant regions are known, and concluded that such regions are characterized by the presence of hydrophobic and hydrophilic residues on opposing faces of the molecule (amphipathic structures). The authors suggested that amphipathic regions exposed by

antigen processing are important for simultaneous interaction of an antigen with Ia (and/or the APC membrane) and the T cell receptor.

Several observations suggest that hydrophobic regions of antigens are important for interaction with APC. Allen *et al* (1984b) localized the immunodominant site of a lysozyme peptide to the 4 amino-terminal residues, but noted that removal of the associated hydrophobic residues abolishes immunogenicity. Thus, these residues may be required to stabilize the association of the peptide with Ia or the APC membrane. The results of Watts *et al* (1985) are also consistent with the notion that a hydrophobic cluster of residues may form the Ia interaction site. Streicher *et al* (1984) described a T cell clone capable of responding to a myoglobin fragment, but not native myoglobin, presented by chloroquine-treated APC. Interestingly, a denatured (but unfragmented) form of the molecule, *S*-methylmyoglobin, could also be presented by chloroquine-treated APC, suggesting that unfolding of myoglobin, rather than fragmentation, is the reason that processing of this antigen is required. Denaturation may expose hydrophobic residues critical for interaction with Ia or the APC membrane.

Another possible explanation of the requirement for antigen processing is that it may be necessary to separate "suppressor" from "helper" determinants present in the native antigen (Goodman and Sercarz, 1983; Krzych *et al*, 1985). Alternatively, processing may be required to expose foreign determinants on an antigen which has few differences in primary structure from the corresponding autologous molecule. This rationale could explain the observation of Berkower *et al* (1982) that T cell populations primed to sperm whale or horse myoglobin (respectively) are completely non-cross-reactive, yet recognize the same immunodominant epitope centered on residue 109. In a similar vein, Schwartz's group has recently proposed that processing of pigeon cytochrome *c* is required to disrupt an ionic bond between glu 62 and lys 99 (Kovač and Schwartz, 1985), so that the latter residue can interact with the T cell receptor (Hansburg *et al*, 1983a).

Although T cells are generally considered to recognize sequential rather than conformational determinants, recent evidence suggests that some aspects of antigen

conformation may affect immunogenicity and antigen processing. Analysis of several protein antigens revealed that many of the immunodominant sites are α-helical in their native configuration (DeLisi and Berzofsky, 1985). Critical immunodominant residues which are not adjacent in the primary sequence may be juxtaposed on the surface of the native molecule by successive turns of an α-helix (Berkower *et al*, 1982). Alternatively, α-helical structures may present a hydrophobic surface on one side of the antigen to stabilize interaction with APC (Watts *et al*, 1985). For antigens which lack α-helical regions in their native configuration, antigen processing may induce or stabilize α-helical structures (DeLisi and Berzofsky, 1985). A recent study by Boyer *et al* (1986) affirms this idea with the demonstration that a synthetic antigen with an α-helical conformation does not require processing to be immunogenic.

An α-helical configuration was shown to be important for the immunogenicity of the C-terminal peptide of various cytochromes *c* (Pincus *et al*, 1983; Schwartz *et al*, 1985). The antigenic determinant of cytochrome *c* is located in the C-terminal 97-104 fragment, yet a larger (88-104) fragment is required for full antigenic potency. Using synthetic cytochrome *c* fragments of various lengths, a good correlation was found between antigenic potency and the percentage of α-helix in the peptide (Schwartz *et al*, 1985). These observations suggest a structural basis for previous observations that a minimum number of amino acids is required for an antigen to be immunogenic (Singh *et al*, 1980). Residues distant from the actual antigenic determinant can also affect antigenic potency by determining the secondary structure adopted by the immunodominant portion of the antigen (Schwartz *et al*, 1985), or by causing differential antigen processing of the fragments containing the immunodominant region (Shastri *et al*, 1986).

Tertiary structure may also be important in the immunogenicity and processing of some antigens. For example, for H-2^b mice, the immunodominant epitope of beef insulin is contained in residues 8-10 of the A-chain (Keck, 1975), yet residues 6-11, which form a disulphide-bonded loop, are required for immunogenicity (Singh and Fraga, 1981). Similarly, some T cell hybridomas from (a x d)F_1 mice were shown to recognize a conformational determinant on pork insulin consisting of a portion of the B-chain and A-chain loop region

(Glimcher *et al*, 1983b). The immunodominant site of native pigeon cytochrome *c* provides another example of an antigen whose immunodominant residues are separated in the primary sequence, but appear to be contiguous on the surface of the molecule in its native configuration (Solinger *et al*, 1979). Büchmuller and Corradin (1982) suggested that conformation could also affect the manner in which an antigen is processed, since, in contrast to most antigens, T cell cross-reactivity for native and denatured cytochrome *c* could not be demonstrated. This was attributed to the fact that unlike most proteins, cytochrome *c* is not denatured at lysosomal pH (4.5) and therefore might be processed differently than cytochrome *c* that is denatured before uptake by APC.

Interleukin-1 Production

IL-1 was described as a MØ-derived lymphokine that promotes lectin-induced thymocyte proliferation (Gery and Waksman, 1972; Gery *et al*, 1972). Since its initial characterization, a number of cellular sources of IL-1 have been identified, including MØ-like lines (Mizel *et al*, 1978a), dendritic cells (Duff *et al*, 1985), B cells (Scala *et al*, 1984b; Gerrard and Volkman, 1985; Matsushima *et al*, 1985; Pistoia *et al*, 1986), natural killer cells (Scala *et al*, 1984a), and many others (reviewed in Durum *et al*, 1985; Oppenheim *et al*, 1986). The biological activity of IL-1 is not restricted to cells of the immune system. For example, IL-1 induces fever, osteoclast proliferation, and is involved in inflammation and wound healing (reviewed in Oppenheim *et al*, 1986). It is not known whether the pleiotropic effects of IL-1 are all mediated by a single molecule, since several biochemical species of IL-1 have been described, and at least two distinct but related IL-1 genes have been identified (reviewed in Oppenheim *et al*, 1986).

Most relevant in terms of A cell function is the participation of IL-1 in T and B cell activation. An obligatory role for IL-1 in T cell activation is suggested by studies demonstrating that IL-1 restores the ability of ultraviolet (UV)-irradiated (Germain, 1981; DeFreitas *et al*, 1983) or fixed (Scala and Oppenheim, 1983) APC to present antigens for T cell activation. Similarly, some Ia$^+$ APC cell lines incapable of IL-1 secretion only activate

antigen-specific T cells (Cohen and Kaplan, 1983) or stimulate allogeneic MLR (Glimcher *et al*, 1982b) in the presence of exogenous IL-1. These studies also support a two-signal model for T cell activation, in which recognition of processed antigen plus Ia (or allogeneic Ia) constitutes the first (specific) signal and IL-1 provides a second (nonspecific) signal. However, other studies have demonstrated that IL-1 is not always required for T cell activation, but merely enhances the response (Glimcher *et al*, 1982b; McKean *et al*, 1985b). Furthermore, T cells may be heterogeneous in their requirement for IL-1, since some T cell clones can be activated by B lymphoma cells incapable of IL-1 secretion (McKean *et al*, 1985a). T cell hybridomas also appear to be IL-1 independent, since they can be activated by antigen on fixed APC (Chestnut *et al*, 1982a; Rock and Benacerraf, 1983b; Shimonkevitz *et al*, 1983, 1984; Allen and Unanue, 1984b; Falo *et al*, 1985; Kovač and Schwartz, 1985). The description of a membrane-bound form of IL-1 on normal MØ (Kurt-Jones *et al*, 1985a), MØ-like tumors (Zlotnik *et al*, 1985), B cells (Kurt-Jones *et al*, 1985b) and dendritic cells (Nagelkerken and van Breda Vriesman, 1986) complicates matters somewhat, since the possible effect of membrane IL-1 was not evaluated in studies demonstrating IL-1 independence. A recent study assessed the effect of quantitative variations in Ia and membrane IL-1 and found that changes in both parameters affect the magnitude of the T cell response (Kurt-Jones *et al*, 1985c), thus providing further evidence of the importance of IL-1 in T cell activation.

IL-1 has also been implicated in the generation of the T cell repertoire during the intrathymic development. Thymic accessory cells produce IL-1 (Beller and Unaue, 1978; Rock and Benacerraf, 1984c; Gallily *et al*, 1985) and thymocytes proliferate in response to self Ia and IL-1 (Rock and Benacerraf, 1984c). Studies with murine thymus organ cultures support the contention that Ia and IL-1 are involved in thymocyte proliferation, since anti-Ia-induced inhibition of proliferation can be restored by the addition of IL-1 (DeLuca, 1985).

In general, the synthesis and secretion of IL-1 by A cells is not constitutive, but is inducible by a wide variety of stimulants (reviewed in Oppenheim *et al*, 1986). The most physiologically relevant stimuli are likely to be Ia-restricted interaction with T cells (Mizel *et al*, 1978a), and stimulation by various microbial products, most notably LPS (Mizel *et al*, 1978b).

T cell recognition of Ia may cause IL-1 release directly, by signal transduction through the Ia molecule, or indirectly, by activating T cells to release IL-1-inducing lymphokines (Moore *et al*, 1980; Dinarello and Kent, 1985). Durum and Gershon (1982) have proposed that the requirement for Ia-restriction of T_h and T_p activation reflects the signal-transducing function of the Ia molecule and the T cell requirement for IL-1, since they found that IL-1 could completely reconstitute antigen-specific T cell proliferation in A cell-depleted T cells. The inhibition of IL-1-dependent T cell mitogenic responses by anti-Ia antibody is also consistent with this idea (Durum *et al*, 1984; Bekoff *et al*, 1985; Smith *et al*, 1985). Furthermore, LPS-induced release of IL-1 from MØ is inhibitable by anti-Ia antibody (Durum *et al*, 1984), and some monoclonal anti-Ia antibodies directly stimulate IL-1 production by human monocytes (Palacios, 1985). Although the Ia molecule may serve a signal-transducing function for A cells, the Ia-restricted recognition of antigen by T cells probably also imparts a activation signal to the T cell through the T cell receptor (Nisbet-Brown *et al*, 1985).

The proliferation of antigen- or lectin-activated T cells is absolutely dependent upon the presence of IL-2, an autocrine growth factor produced by T cells (reviewed in Smith, 1984). Some studies have shown that IL-1 stimulates or enhances IL-2 production by T cells (Larsson *et al*, 1980; Smith *et al*, 1980). Others ascribe the effects of IL-1 in T cell activation to the induction of IL-2 receptors on activated T cells (Kaye *et al*, 1983; Kaye and Janeway, 1984; Männel *et al*, 1985), although the acquisition of responsiveness to IL-2 in the absence of IL-1 has also been demonstrated (Katzen *et al*, 1985; Tsoukas *et al*, 1985). Clearly, more study is required to determine the cellular basis of IL-1 activity in T cell proliferation. At the molecular level, the interaction of IL-1 with its receptor may transduce a signal to the T cell *via* the protein kinase C pathway, since phorbol myristate acetate, an activator of this enzyme (Castagna *et al*, 1982), can substitute for IL-1 (Rosenstreich and Mizel, 1979).

IL-1 has also been implicated as a cytokine for antigen-specific and polyclonal B cell activation (Corbel and Melchers, 1983; Falkoff *et al*, 1983; Lipsky *et al*, 1983; Howard *et al*, 1983; Pike and Nossal, 1985; Sinha *et al*, 1986). Both proliferative and differentiative functions have been described (Booth *et al*, 1983; Howard and Paul, 1983; Lipsky *et al*, 1983; Pike and

Nossal, 1985). The involvement of IL-2 in B cell activation (reviewed in Howard *et al*, 1984), though still controversial, raises the possibility that IL-1 may function identically in B and T cell activation. Although the exact role of IL-1 in B cell activation remains to be established, Sinha *et al* (1986) have shown that contrary to earlier suggestions (Letvin *et al*, 1981; Morrissey *et al*, 1981), IL-1 secretion is the only A cell function required for the activation of B cells by TI antigens. Thus, B cells do not need to recognize processed antigen on APC.

Other Accessory Cell Functions

Purified T cells can respond to mitogens such as Con A or PHA with the cooperation of non-MHC-compatible A cells (Lipsky *et al*, 1976; Habu and Raff, 1977; Rosenstreich and Mizel, 1978; Kammer and Unanue, 1980), suggesting that self MHC-restricted antigen presentation is not a requirement for mitogenic responses. IL-1 secretion is clearly important in these systems (Rosenstreich *et al*, 1976; Kammer and Unanue, 1980), but other A cell functions are probably involved as well, since IL-1 seldom reconstitutes these responses fully (Bekoff *et al*, 1985; Davis and Lipsky, 1985). Much better reconstitution of the Con A response is achieved with IL-1 in the presence of fixed A cells (Bekoff *et al*, 1985). Moreover, a recent paper by Roska and Lipsky (1985) indicates that although MHC-restricted antigen presentation and IL-1 secretion are necessary for antigen-specific T cell responses, they are not sufficient. Thus, optimal T cell responses to antigens and mitogens may require direct, non-MHC-restricted A cell-T cell contact, in addition to MHC-restricted antigen presentation or IL-1 secretion. Promotion of lymphocyte viability is an additional nonspecific A cell function, but this does not require cell-cell contact (Pierce *et al*, 1974).

Despite the absence of Ia restriction in mitogen-induced T cell activation, Ia^+ A cells were reported to function more efficiently than Ia^- A cells (Ahmann *et al*, 1978; Kammer and Unanue, 1980; Rock, 1982) and anti-Ia antibody substantially inhibits the response in the presence of Ia^+ A cells (Durum *et al*, 1984; Bekoff *et al*, 1985; Smith *et al*, 1985). The involvement of the L3T4 molecule in this A cell-T cell interaction may depend on the particular mitogen used, since anti-L3T4 inhibits sodium periodate-induced (Smith *et al*, 1985) but not

Con A-induced (Bekoff *et al*, 1985) T cell mitogenesis in the presence of Ia$^+$ A cells. Interestingly, anti-L3T4 antibody is inhibitory for the Con A response only when Ia$^-$ L cells are used as A cells (Bekoff *et al*, 1985). This may indicate that additional non-MHC-restricted A cell-T cell interactions mediated by nonpolymorphic Ia determinants (or other molecules), are necessary for optimal antigen and mitogen responsiveness. The nature of the signal delivered by this interaction has not been defined.

C. Accessory Cell Heterogeneity

The cell type which performs critical accessory functions at the initiation of most, if not all, immune responses was initially identified as a MØ (reviewed in Unanue, 1972, 1981, 1984) by a number of functional criteria, such as adherence (Mosier, 1967; Unanue, 1972), radiation resistance (Roseman, 1969; Gorzynski *et al*, 1971), and phagocytic capacity (Shortman *et al*, 1970; Pierce *et al*, 1974). However, there have since been many reports that other cells have A cell activity. Steinman and co-workers isolated a novel type of bone marrow-derived cell from murine lymphoid organs, which is distinguishable from MØ by its dendritic morphology, absence of FcR, low phagocytic capacity, and a number of other criteria summarized in Table 1 (reviewed in Steinman and Nussenzweig, 1980, Steinman, 1981). Such lymphoid dendritic cells were shown by Steinman's group and others to have potent A cell activity for a variety of *in vitro* immune responses (Nussenzweig *et al*, 1980; Sunshine *et al*, 1980, 1983; Röllinghoff *et al*, 1982). Of great importance to the understanding of antigen-specific, MHC-restricted collaboration between T and B cells is the recent demonstration that under certain conditions, B cells can process and present antigens to T cells (Chestnut and Grey, 1981; reviewed in Chestnut and Grey, 1985). Perhaps most unexpected was the discovery that many nonlymphoid cell types, such as endothelial cells (Hirschberg *et al*, 1980; Burger *et al*, 1981), fibroblasts (Lipsky and Kettman, 1982; Umetsu *et al*, 1985) and epithelial cells (Londei *et al*, 1984) can function as A cells for some types of *in vitro* immune responses. While the importance of nonlymphoid A cells in normal immune responses remains to be established, it has been speculated that these cells may be important in the pathogenesis of

TABLE 1

Cellular Characteristics of MØ and DC

Characteristic[a]	MØ	DC
A. Morphology		
cytoplasm	- form processes only following adherence	- have bulbous cytoplasmic processes when adherent or free in suspension
	- surface ruffling	- continuous formation and retraction of cell processes
nucleus	- oval, remains sedentary	- irregularly shaped, undergoes pulsatile movement
organelles	- many pinocytic vesicles and lysosomes	- few lysosomes
	- rod-like mitochondria	- spherical phase dense mitochondria
B. Surface Antigens and Receptors	- Ia inducible and variable	- constitutively Ia^+
	- FcR^+	- FcR^-
C. Adherence	- strongly adherent	- transiently adherent, fail to readhere once dislodged
D. Phagocytosis	- highly phagocytic, increased by activation	- nonphagocytic

[a]Reference - Steinman and Nussenzweig, 1980.

certain autoimmune diseases (Bottazzo *et al*, 1983).

The realization that A cell functions can be performed by a variety of cell types has made it difficult to assess their relative importance in immune responses *in vivo* and *in vitro*. Furthermore, some A cell types may only possess a certain subset of all the A cell functions that have been described, and may therefore be active in some assays but not others. In the following sections, the functional characteristics of several A cell types will be reviewed, with particular emphasis on MØ, DC, and B cells, the primary A cells in lymphoid organs. The use of homogeneous tumor lines with A cell activity and artificial systems for investigating A cell function will also be discussed.

Macrophages

Macrophages display considerable heterogeneity with respect to surface Ia content (reviewed in Unanue, 1984), size (Gordon and Cohn, 1973; Rice and Fishman, 1974), quantity and class of FcR (Walker, 1974; 1976; Rhodes, 1975), and phagocytic ability (Zembala and Asherson, 1970; Rice and Fishman, 1974). In addition, many different functions have been attributed to MØ, including A cell activity for immune responses (reviewed in Unanue, 1972, 1981, 1984), cytostatic and cytotoxic activity against tumors (reviewed in Lohmann-Matthes and Fischer, 1973; Levy and Wheelock, 1974; Shin *et al*, 1978), and secretion of a multifarious array of inflammatory mediators (reviewed in Allison, 1978; Unanue, 1981; Adams and Hamilton, 1984). Inflammatory or immunological activation stimuli enhance, and are sometimes required for the expression of many of these functional capacities (reviewed in Cohn, 1978; Karnovsky and Lazdins, 1978; North, 1978; Adams and Hamilton, 1984).

In view of the diversity of MØ phenotypes and functions, it is not surprising that their role in specific immune responses has been controversial. Although the A cell in many systems has been shown to have MØ-like properties (reviewed in Unanue, 1972, 1981, 1984), MØ have also been reported to suppress mitogen- or antigen-induced lymphocyte proliferation (Scott, 1972; Yoshinaga *et al*, 1972; Folch *et al*, 1973; Kirchner *et al*, 1975b; Lipsky and Rosenthal, 1976; Pope *et al*, 1976; Kurland *et al*, 1977; Rosenstreich and Mizel, 1978), *in vitro* AFC

responses (Diener *et al*, 1970; Hoffmann, 1970; Sjöberg, 1972; Lemke *et al*, 1975; Lee and Berry, 1977), and *in vitro* cell-mediated immune responses (Lonai and Feldmann, 1971; Kirchner *et al*, 1975a). While it is possible that the many functions attributed to MØ are mediated by one multifunctional cell type, it is more likely that functionally distinct MØ subsets subserve various functions (Walker, 1971, 1974, 1976; Rice and Fishman, 1974; Lee and Berry, 1977; Serio *et al*, 1979; Lee, 1980a; Lee and Wong, 1980, 1982). Such heterogeneity could result from distinct MØ lineages (Bursuker and Goldman, 1983) or functional differentiation within a single lineage. The latter possibility is supported by most evidence (reviewed in Lee, 1980a) as will be discussed following a brief description of MØ differentiation.

The *in vitro* (and presumably also *in vivo*) growth and differentiation of MØ is critically dependent on MØ growth factor, also known as colony-stimulating factor-1 (CSF-1) (Metcalf and Moore, 1973). MØ differentiate in a linear sequence leading from actively dividing bone marrow precursors (monoblasts and promonocytes) to non-dividing peripheral blood monocytes (van Furth and Cohn, 1968). After an average period of about 30 hr in the peripheral circulation, monocytes leave the blood and complete their maturation in various tissues (reviewed in Steinman and Cohn, 1974). However, not all tissue MØ are derived from circulating monocytes. In the mouse, about 55% of splenic MØ are derived from monocytes, whereas the other 45% are descendents of a proliferating splenic precursor (van Furth and Disselhoff-den Dulk, 1984). Whether the MØ derived from the two sources represent functionally distinct lineages has not been determined. The maturation of blood monocytes into MØ occurs without cell division (van Furth and Cohn, 1968) but with a substantial (3-fold) increase in cell size (reviewed in Gordon and Cohn, 1973; Steinman and Cohn, 1974). The level of peroxidase in MØ granules serves as a useful cytochemical marker of MØ differentiation, since MØ maturation is associated with a decrease in peroxidase activity (van Furth *et al*, 1970; Simmons and Karnovsky, 1973).

Mature MØ reside in many different lymphoid and nonlymphoid tissues, including the spleen, lymph nodes, thymus, bone marrow, peritoneal cavity, lungs, kidneys, and liver (Lee *et*

al, 1985). The percentage of MØ and the proportion that are Ia$^+$ (and therefore capable of functioning as APC) vary greatly among different tissues (reviewed in Unanue, 1984). Resident peritoneal cells contain about 40% MØ that are predominantly mature, as evidenced by their low peroxidase content (Lee and Berry, 1977). Although unactivated peritoneal MØ have APC activity (Lee *et al*, 1979b; Ziegler and Unanue, 1979), only activated MØ express tumor cytolytic activity (reviewed in Adams and Hamilton, 1984). Size-fractionation of activated peritoneal cells by velocity sedimentation reveals that APC activity is associated primarily with small (less mature) MØ (despite substantial numbers of Ia$^+$ cells in the large activated MØ), whereas the anti-tumor acitivity is mediated predominantly by large activated MØ (reviewed in Lee, 1980a). The enhanced nonspecific suppressive activity of activated MØ is also concentrated in the large MØ (Lee and Berry, 1977). Activated and size-fractionated culture-derived bone marrow (BM)-MØ display similar functional heterogeneity (Lee and Wong, 1980, 1982). Moreover, large activated BM-MØ are nonspecifically suppressive for antigen-specific T cell proliferation, which may explain their poor APC activity. Collectively, these observations suggest that APC activity is only expressed transiently during the early phase of MØ differentiation, and is lost when MØ are large and fully mature (Lee, 1980a).

Unlike the predominantly Ia$^-$ peritoneal MØ (Yamashita and Shevach, 1977; Cowing *et al*, 1978b), the majority of splenic MØ are Ia$^+$ (Cowing *et al*, 1978b), small (Lee, 1980b), and immature (Steinman *et al*, 1979), and have thus been a popular source of APC (Cowing *et al*, 1978a; Schwartz *et al*, 1978). However, it should be emphasized that the major (and usually only) criterion for purification of splenic MØ in most of these earlier studies was adherence, and although these splenic adherent cells were enriched for phagocytic cells, nonphagocytic DC were probably present as well. Very few studies have assessed the ability of highly purified splenic MØ to present xenogeneic antigens for T cell activation, although Steinman and co-workers reported that splenic MØ are ineffective A cells for the *in vitro* activation of primary anti-TNP CTL (Nussenzweig *et al*, 1980). Most studies with purified splenic MØ have assessed A cell function in terms of allogeneic MLR stimulation, with contradictory results. Steinman *et al* (1983) found that splenic (as well as peritoneal) MØ are virtually inactive in this

regard, whereas Minami *et al* (1980), and Sunshine *et al* (1982) obtained good allogeneic MLR stimulation by splenic MØ. The reasons for this discrepancy are not clear, but in view of Steinman's contention that all of the MLR-stimulating capacity in MØ preparations can be attributed to contaminating DC (Steinman *et al*, 1983), this matter will not be fully resolved until a source of MØ free from DC is assessed for the potential to activate MLR.

Humphrey has identified two types of splenic MØ, according to their localization within the spleen, Ia expression, and selective retention of different polysaccharides (Humphrey, 1981; Humphrey and Grennan, 1981). Marginal zone MØ are large, predominantly Ia^-, selectively trap ficoll and starch, often form clusters with B cells, and are only released upon collagenase treatment of spleen cells. In contrast, red pulp MØ are small, mostly Ia^+, selectively trap pneumococcal polysaccharides, and do not cluster with B cells. The Ia positivity and immaturity of the red pulp MØ makes them the most likely candidates for APC, although this was not evaluated in these studies.

Macrophages from several other tissues have also been assessed for APC function. Thymic MØ are 50-75% Ia^+ and can present *L. monocytogenes* to *Listeria*-specific T cell lines (Beller and Unanue, 1980). Alveolar MØ have been reported to be efficient (Holt and Batty, 1980; Lipscomb *et al*, 1981) or inefficient (Ullrich and Herscowitz, 1980; Weinberg and Unanue, 1981) APC, according to different studies. These discrepant reports may be due to variation in the levels of Ia^+ alveolar MØ in different species, ranging from 5% in mice (Weinberg and Unanue, 1981) to 80% in guinea pigs (Lipscomb *et al*, 1981) and humans (Toews *et al*, 1984). Moreover, there have been several reports that alveolar MØ are suppressive (Herscowitz *et al*, 1979; Holt and Batty, 1980; McCombs *et al*, 1982; Toews *et al*, 1984) probably due to prostaglandin secretion (Schulyer and Todd, 1981). Hepatic MØ (Kuppfer cells) are 20-50% Ia^+ and present antigen (Richman *et al*, 1979; Rogoff and Lipsky, 1980), support mitogen-induced T cell proliferation (Rogoff and Lipsky, 1979), and stimulate MLR (Nadler *et al*, 1980). Finally, Ia^+ renal mesangial MØ are capable of antigen presentation (Schreiner *et al*, 1981). Thus, most organs contain Ia^+ MØ that have APC activity *in vitro*, and these may be important *in vivo* in the pathogensis of autoimmune diseases.

Regulation of Ia Expression on Macrophages

Ia expression on different MØ populations varies greatly, and is subject to both positive and negative regulation by soluble factors. Prostaglandins (Snyder *et al*, 1982), corticosteroids (Snyder and Unanue, 1982), and α-fetoprotein (Lu *et al*, 1984) down-regulate surface Ia on MØ, whereas secreted products of activated T cells (Scher *et al*, 1980; Steinman *et al*, 1980; Steeg *et al*, 1981), at least one of which is interferon-γ (IFN-γ) (Steeg *et al*, 1982), enhance it. Consequently, the percentage of Ia^+ MØ depends on the immune status of the animal. However, maintenance of the low basal level of Ia^+ MØ is apparently T cell-independent, since athymic mice are not deficient in this regard (Lu *et al*, 1981).

Regulation of Ia expression *in vivo* has been extensively studied with peritoneal MØ. Various inflammatory stimuli administered intraperitoneally, such as thioglycolate, oil, and peptone, stimulate an influx of MØ into the peritoneal cavity, but the ratio of Ia^+/Ia^- MØ does not change (Beller *et al*, 1980). In contrast, intraperitoneal injection of immunological stimuli, including microorganisms (Beller *et al*, 1980; Nussenzweig *et al*, 1980; Behbehani *et al*, 1981) and adjuvants (Behbehani *et al*, 1985), greatly increase the proportion of Ia^+ MØ. The radiosensitivity of the increase in Ia^+ MØ (Scher *et al*, 1982) suggests that it is due to recruitment and conversion of Ia^- proliferating percursors rather than conversion of resident Ia^- peritoneal MØ to Ia^+ MØ. The enhancement of Ia expression is relatively transient and disappears after 48 hr in culture in the absence of lymphokines (Steinman *et al*, 1980; Beller and Unanue, 1981). In contrast, Ia expression on resident peritoneal MØ appears to be relatively stable (Walker *et al*, 1983).

Dendritic Cells

Dendritic cells were initially identified by their unusual morphology in murine lymphoid cell suspensions (reviewed in Nussenzweig and Steinman, 1980; Steinman, 1981), and have since been described in humans (Kuntz Crow and Kunkel, 1982; Van Voorhis *et al*, 1982) and rats (Klinkert *et al*, 1980, 1982), with minor differences. Notably, human DC possess complement receptors (CR) (Van Voorhis *et al*, 1982) and rat DC are nonadherent (Klinkert *et al*, 1980,

1982). Murine DC are only transiently adherent; they detach spontaneously after overnight culture, and unlike MØ, will not readhere.

The constitutive expression of high levels of Ia (reviewed in Nussenzweig and Steinman, 1982) makes DC good candidates for APC. Accordingly, murine splenic DC were shown to stimulate allogeneic MLR (Steinman and Witmer, 1978; Sunshine *et al*, 1982), support primary *in vitro* AFC responses (Inaba *et al*, 1983), and present antigens to T cells in an MHC-restricted manner. The extreme potency of even small numbers of DC in stimulating allogeneic MLR suggests that they may be important in evoking allograft rejection. This notion has recently received experimental support (Lechler and Batchelor, 1982; Faustman *et al*, 1984).

DC have the unique capacity to elicite a proliferative response in syngeneic T cells (syngeneic MLR), whereas monocytes and MØ are very inefficient in this regard (Nussenzweig and Steinman, 1980; Van Voorhis *et al*, 1983). The thymus determines the specificity of the syngeneic MLR (Glimcher *et al*, 1982c), and the responding T cells recognize syngeneic Ia in the apparent absence of xenogeneic antigens (Glimcher *et al*, 1981). The bromodeoxyuridine and light suicide technique revealed that the T cells proliferating in the syngeneic MLR are a subset of those that proliferate in response to antigen and syngeneic Ia (Dos Reis and Shevach, 1981). Furthermore, some antigen-specific T cell clones and hybridomas can be activated by syngeneic APC in the absence of antigen (Rock and Benacerraf, 1983c; Dos Reis and Shevach, 1985). The ability of some T cells to be activated by syngeneic Ia alone contradicts immunological dogma that such cells should not exist in normal individuals. Unclear at present is whether the syngeneic MLR is an *in vitro* artefact, perhaps due to the removal of T cells from critical *in vivo* immunoregulatory influences, or has some as yet unappreciated physiological relevance.

Epidermal Langerhans cells (LC) are morphologically similar to DC, nonphagocytic, and contain characteristic Birbeck granules, although the similarity of their surface phenotype (FcR^+, CR^+) to that of MØ led to suggestions that they are part of the mononuclear phagocyte system (Stingl *et al*, 1980). However, it has recently been shown that LC mature into DC *in vitro*, suggesting that DC and LC belong to the same lineage of cells (Schuler and Steinman,

1985). Like DC, LC are strongly Ia$^+$ and can present antigen and stimulate allogeneic and syngeneic MLR *in vitro* (Stingl *et al*, 1978; Bienenstock *et al*, 1985). Several studies indicate that LC are the primary APC *in vivo* for skin hypersensitivity responses (reviewed in Silberberg-Sinakin *et al*, 1980), although Ia$^+$ skin keratinocytes may also be functional in this regard (Roberts *et al*, 1985). Interestingly, if LC are depleted by UV irradiation of the skin, exposure to contact allergens leads to specific unresponsiveness (Toews *et al*, 1980).

Other cells with certain phenotypical and morphological similarities to LC and lymphoid DC have been described, but their developmental relationship to DC and LC has not been established (reviewed in Tew *et al*, 1982). Thymic dendritic cells are Ia$^+$ (Rouse *et al*, 1979) and have APC function *in vitro* (Kyewski *et al*, 1984, 1986). Veiled cells, which are present in the afferent lymph draining the skin, are similar to LC in surface phenotype, and some contain Birbeck granules. The APC function of these cells has not been fully assessed, but they do serve as accessory cells for Con A- or heterologous Ig-induced lymphocyte proliferation (Balfour *et al*, 1981; Knight *et al*, 1982). The T-dependent area (paracortex) of lymph nodes contains dendritic-like interdigitating cells which may be derived from veiled cells. They express Ia (Lampert *et al*, 1980) and thus may have APC function, but this has not been tested. Since interdigitating cells have been identified only in tissue sections of lymphoid organs, their relationship to lymphoid DC is not established, but they may be identical. Finally, cells with dendritic morphology have been identified histologically in the follicles (B cell areas) of lymph node and spleen (reviewed in Klaus *et al*, 1980; Mandel *et al*, 1980). These FcR$^+$, CR$^+$ follicular DC have been shown to be responsible for trapping and retention of immune complexes in lymphoid organs, and may be involved in the generation of immunological memory. Originally, they were reported to be Ia$^-$ (Barclay, 1981; Humphrey and Grennan, 1982), but later studies have reported follicular DC as Ia$^+$ (Gerdes *et al*, 1983; Kosco *et al*, 1985; Schnizlein *et al*, 1985). Follicular DC have recently been isolated in suspension (Schnizlein *et al*, 1985); thus, their APC potential should soon be known.

B Cells

"Antigen-bridge" models were initially invoked to account for observations that T_h and B cells are required to recognize determinants on the same molecule (Raff, 1970; Mitchison, 1971; Rajewsky, 1971). However, the subsequent demonstration by Katz *et al* (1973) that T_h-B cell interaction is I region-restricted made this model untenable, since it was not obvious how T_h could simultaneously recognize antigen and Ia if the antigen was engaged in bridging T_h and B cells *via* their specific receptors. Central to the resolution of this conundrum was the observation of Chestnut and Grey (1981) that B cells can process and present antigens to T cells in an MHC-restricted manner. Linked associative recognition is thus a manifestation of the fact that B cells efficiently present only those antigens for which they are specific, as will be discussed in the following paragraphs.

The capacity of B cells to serve as efficient APC is a direct consequence of their ability to concentrate antigen *via* antigen-specific Ig receptors (Chestnut *et al*, 1982b; Kakiuchi *et al*, 1983; Rock *et al*, 1984; Lanzavecchia, 1985). Although activated B cells (Chestnut *et al*, 1982b; Kakiuchi *et al*, 1983) and several B cell tumors (McKean *et al*, 1981; Chestnut *et al*, 1982a,b; Glimcher *et al*, 1982a,b; Issekutz *et al*, 1982; Kappler *et al*, 1982; Walker *et al*, 1982b) effectively present many protein antigens without participation of their Ig receptors, this may be due to the greater pinocytic activity of these cells as compared to normal resting B cells (Chestnut *et al*, 1982b). Furthermore, resting human B cells have been reported to be less efficient at processing antigen, because, in contrast to monocytes, antigen-pulsed resting B cells are not capable of specifically binding T cells (Brozek *et al*, 1984). Consistent with the proposed role of Ig receptors in increasing the efficiency of antigen uptake, normal B cells exhibit much greater uptake of an antigen that is capable of binding to its Ig receptors (Chestnut *et al*, 1982b; Kakiuchi *et al*, 1983). In addition to facilitating antigen uptake, the binding of some antigens to surface Ig may activate the B cell such that it becomes a more effective APC (Kakiuchi *et al*, 1983, Casten *et al*, 1985) by virtue of its increased pinocytic activity or increased Ia expression (Mond *et al*, 1981; Monroe and Cambier, 1983). However, Ia density is not always positively correlated with APC function (Walker *et al*, 1982a).

Therefore, the increased Ia expression following B cell activation may not be critical to the APC function of the B cell. Moreover, since activated B cells are also more efficient than resting B cells in supporting the Con A response (Krieger *et al*, 1985) factors other than increased pinocytic activity or increased Ia expression must be relevant to the greater efficiency of activated B cells as APC.

Unlike the APC function of MØ and DC, the APC function of resting B cells is ablated by high (> 3000 rad) doses of γ-irradiation (Ashwell *et al*, 1984). This may explain why some investigators have failed to observe B cell APC function (Bandeira *et al*, 1983). B cell activation results in the acquisition of radioresistance (Frohman and Cowing, 1985; Krieger *et al*, 1985), although the appearance of APC function and radioresistance following activation are temporally dissociated (Krieger *et al*, 1985). The radiosensitivity of B cell APC function may be due to the dual requirement for activation and antigen processing, both of which may be adversely affected by radiation (Abbas *et al*, 1985).

The Ig receptor is involved only in the uptake of antigen, and plays no role in antigen presentation, since antigen uptake, but not presentation, is inhibitable by anti-Ig antibody (Abbas *et al*, 1985; Lanzavecchia, 1985). Like MØ, B cells process antigen into immunologically relevant moieties *via* a chloroquine-sensitive pathway (Chestnut *et al*, 1982a; Lanzavecchia, 1985), and these two cell types display similar degradative capacities for a soluble protein antigen (Grey *et al*, 1982). At extremely low antigen concentrations, B cells are the most effective APC by virtue of their specific antigen receptors, which serve to specifically concentrate antigen (Rock *et al*, 1984; Abbas *et al*, 1985; Lanzavecchia, 1985; Malynn *et al*, 1985). One study reported that splenic adherent cells were 4 times more effective than B cells as APC, but purified antigen-specific B cells were not used (Frohman and Cowing, 1985).

Activated B cells (Chestnut and Grey, 1985) and some B cell tumors (Glimcher *et al*, 1982a,b) also stimulate allogeneic MLR, although this ability is dependent on the addition of exogenous IL-1. B cell production of IL-1 has been reported (reviewed in Oppenheim *et al*, 1986), but not all B cells may be equivalent in this capacity. Chu *et al* (1985) reported that B cells transformed by Epstein Barr virus were IL-1 deficient and failed to present antigen to

resting T cells. However, it is difficult to assess the effects of IL-1 in such systems, since it may augment the function of B cells independently of its effect on T cells (Kakiucki *et al*, 1983).

Activation of T cells by antigen presented on B cells *vs* MØ or DC may not be functionally equivalent. Ashwell *et al* (1984) found that some T cell clones proliferate to antigen presented on B cells, whereas other clones produce B cell activating factors without proliferating. Similarly, McKean *et al* (1985b) reported that some B lymphoma APC could not induce antigen-specific T cell proliferation, despite the stimulation of IL-2 release. DeKruyff *et al* (1986) found that some T cell clones could not be activated by antigen on B cells, and Minami *et al* (1985) described B cell-reactive and B cell-nonreactive alloreactive T cell hybridomas. Cullen *et al* (1981) demonstrated that MØ and B cell Ia molecules are differentially glycosylated; this could provide a biochemical basis for T cell discrimination between the two APC types (Cowing and Chapdelaine, 1983).

What is the importance of B cell APC function to the immune system? Chestnut and Grey (1985) proposed that initially, MØ would activate T cells, since they greatly outnumber the antigen-specific B cells capable of presenting a particular antigen. Once T cells have been activated and clonally expanded by MØ-associated antigen, the likelihood of interacting with specific B cells which display processed antigen on their surface is much greater. This antigen-specific, MHC-restricted interaction then leads to B cell activation. Thus, the APC function of B cells is crucial to the regulation and specificity of B cell activation. Activated B cell could then amplify the T cell response by presenting other antigens nonspecifically.

Other Antigen-Presenting Cell Types and Artificial Antigen-Presenting Systems

Other Ia^+ cells that can function as APC under certain circumstances are natural killer cells (Scala *et al*, 1985), T cells (Forre *et al*, 1982; Sopori *et al*, 1985; Reske-Kunz *et al*, 1986), and neutrophils (Fitzgerald *et al*, 1983). Furthermore, IFN-γ induces Ia expression and APC function in a variety of Ia^- cell types, including astrocytes (Wong *et al*, 1984; Fierz *et al*, 1985), endothelial cells (Wagner *et al*, 1984), and some MØ tumor lines (Birmingham *et al*, 1982;

Walker *et al*, 1982a). The acquisition of APC function by L cells (Malissen *et al*, 1984; Norcross *et al*, 1984) and B cell lymphomas (Germain *et al*, 1983; Ben-Nun *et al*, 1984; Folsom *et al*, 1984) transfected with class II MHC genes, or fused with Ia-bearing liposomes (Coeshott and Grey, 1985), suggests that any cell expressing Ia can function as an APC. This minimalist view of APC function has been tested in several ways. Walden *et al* (1985) inserted class II MHC molecules into synthetic lipid vesicles which contained OVA or beef insulin covalently linked to the lipid, and found that such vesicles activate cloned T cells and hybridomas in an antigen- and MHC-specific manner. However, this mode of T cell stimulation was not compared to stimulation by antigen and conventional APC, making it difficult to assess the significance of these results. Watts *et al* (1984) found that planar lipid membranes, but not liposomes, containing purified I-A^d and a noncovalently associated OVA fragment, stimulate IL-2 production from T cell hybridomas, but this mode of stimulation is much less efficient than activation by antigen and fixed APC (Watts *et al*, 1985). This could reflect the important stabilizing influence of accessory molecules such as LFA-1, the ligand for which is not present in such artificial constructs (Gay *et al*, 1986).

These results suggest that even when IL-1 secretion (since T hybridomas were used) and antigen processing are not considerations, the presence of Ia in a cell membrane is not sufficient to induce maximal T cell activation. Although artificial antigen-presenting systems are valuable for examining the interaction of the T cell receptor with antigen plus Ia, they do not allow examination of the full complement of A cell functions and heterogeneity. This point is well-illustrated by the recent study of Malissen *et al* (1984) demonstrating that Ia-transfected L cells and hamster B cell lines can present KLH, but not OVA, for unknown reasons. Similarly, Shastri *et al* (1985) found that Ia-transfected L cells can present a lysozyme peptide to specific T cells, but not the native protein. This indicates that besides Ia expression, a major factor determining the ability of a cell to function as an APC may be its antigen-processing capacity. However, Ia^+ cells with poor processing function may be able to present processed fragments released from another more capable antigen-processing cell.

D. Rationale and Objectives

The preceeding discussion has documented the substantial progress that has been made concerning our understanding of the nature of antigen processing, the regulation of Ia antigen expression, the specificity of T cells for Ia and processed antigens, and the existence of accessory cell heterogeneity. Nevertheless, several major questions remain. For example, why are there so many A cell lineages? More specifically, why are there several types of specialized APC (MØ, DC, and B cells), whose functions appear to overlap? It is unlikely that one A cell type is omnipotent, but since extensive functional comparsions of different A cell types has not been done, this remains a possibility. Division of labor between various A cells is more likely, so that functionally heterogeneous APC can complement each other for the processing and presentation of a whole range of antigens. To test this hypothesis, cell fractionation and purification techniques were used to obtain very pure MØ and DC populations. Fractionation is particularly important for MØ, which are functionally heterogeneous. B cell APC function was not assessed, since, in preliminary experiments, they were shown not to be involved under the conditions used. MØ and DC were functionally compared for their ability to induce T proliferative responses to various xenogeneic antigens, as well as to syngeneic and allogeneic Ia. The mechanism of antigen processing was investigated using the lysosomotropic agent chloroquine to inhibit lysosomal function. Although the MØ and DC fractions were very pure, cross-contamination is always a consideration when cell purification methods are used. Therefore, the results obtained with purified MØ and DC were confirmed with pure culture-derived BM-MØ and homogeneous cell lines with DC-like or MØ-like properties. Finally, the possibility of cell collaboration between DC and MØ was assessed by using limiting numbers of each cell type to complement each other as APC in T proliferative responses.

II. MATERIALS AND METHODS

Sources of materials. These are listed in Table 2.

Antigens. Polymeric flagellar protein (POL) was prepared according to Ada *et al* (1964). Monomeric flagellin (MON) was prepared by dissociation of POL in 0.05 N HCl immediately before use. *Corynebacterium parvum* (CP, also known as *Propionibacterium acnes* strain 0009) was grown under nitrogen in a standard medium containing Trypticase, glucose, cysteine, Tween 20, yeast extract, hemin, and vitamin K. The bacteria were heat-killed (56° C, 30 min) and stored at 4° C in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) at 7 mg/ml. Lyophilized *Bacillus Calmette Guérin* (BCG) organisms were reconstituted with PBS to a final concentration of 7 mg/ml, heat-killed as described for CP, and stored at 4° C.

Antigens were labeled with ^{125}I by the chloramine T method of Parish and Ada (1969) when required and dialyzed (POL, MON and KLH) or washed (CP) extensively before use. Where indicated, CP was fixed with 2.5% (v/v) glutaraldehyde in PBS for 20 min, neutralized with 0.2 M lysine, and then washed three times before use.

Antibodies. Ascitic fluids were raised with hybridomas secreting anti-I-A^k (10-2.16), anti-I-A^d (MKD6), or and anti-sheep red blood cell (SRBC, N-S.8.1 and S-S.1) monoclonal antibodies. For some experiments, the 16-3-1N, MKD6 and 10-2.16 hybridomas were adapted to grow in defined serum-free medium (DSI medium) so that the supernatants would not contain extraneous IgG and could be used without further purification.

Ia-inducing lymphokines. The lymphokine-containing supernatant of Con A-activated rat spleen cells was used to induce Ia expression on BM-MØ (Glasebrook and Fitch, 1980). Spleen cells (1.25×10^6/ml) from Sprague-Dawley rats were suspended in Dulbecco's modified minimum essential medium (DMEM) containing 2% fetal bovine serum (FBS), 5×10^{-5} M 2-mercaptoethanol (2-ME), and 2.5 μg/ml Con A, and incubated in 500 ml glass bottles (60 ml/bottle) for 48 hr. The cell-free supernatant was then absorbed with Sephadex G-25 (1 g/100 ml supernatant) for 4 hr with gentle stirring at room temperature. The Sephadex was removed by centrifugation and the supernatant absorbed once more with Sephadex G-25

TABLE 2

Sources of Materials

Material	Source
Animals	Health Sciences Animal Center, University of Alberta
Mice	
CBA/CaJ (H-2^k, *Mls*^{b})	
C3H/HeJ (H-2^k, *Mls*^{c})	
BALB/cCR (H-2^d, *Mls*^{b})	
DBA/2J (H-2^d, *Mls*^{a})	
CBA/J (H-2^k, *Mls*^{d})	
Sprague-Dawley Rats	
Antigens, Mitogens and Adjuvants	
BCG	Institute Armand-Frappier
CFA H37Ra, IFA, LPS	Difco Laboratories
Con A, KLH	Calbiochem-Behring
CP	Dr. C.S. Cummins, VA Polytechnic Inst.
OVA	Sigma Chemical Co.
PPD	Connaught Laboratories
POL	prepared from *S. paratyphi* SL1699
S. paratyphi SL1699	Dr. B.A.D. Stocker, Stanford University
Antibodies	
peroxidase-anti-mouse IgG F(ab')$_2$	Tago
Bacto anti-sheep hemolysin	Difco Laboratories
FITC-SAMIG	Cappel
Tissue Culture Medium	
DSI serum-free	Quadralogic Co.
DMEM, HBSS, Medium 199, RPMI	Gibco
FBS, horse serum	Hyclone
gentamicin sulphate	United States Biochemical Corp.
human serum	Canadian Red Cross, Edmonton
penicillin-streptomycin	Flow Laboratories

This table is continued on the next page.

TABLE 2 -- continued

Material	Sources
Tissue Culture Supplies	
24-well plates	Linbro
96-well plates (flat- V-, or round-bottomed)	Linbro
17 x 100 mm centrifuge tubes	Falcon 2001
50 ml polypropylene tubes	Falcon 2070
50 ml polystyrene tubes	Corning
75 cm^2 flasks	Falcon 3024
100 x 20 mm Petri dishes	Falcon 1005
100 x 20 mm tissue culture dishes	Falcon 3003
Hybridomas and Cell Lines	
10-2.16 (αI-A^k, IgG_{2b})	ATCC
16-3-1N (αH-$2K^k$, IgG_{2b})	ATCC
EL4 thymoma	Dr. V. Paetkau, University of Alberta
J774A-1	ATCC
L cells (clone L-929)	Dr. C. Stewart, Los Alamos Nat. Lab.
MKD6 (αI-A^d, IgG_{2b})	ATCC
NKD11	Dr. H. Tse, Merck Institute
N-S.8.1 (αSRBC)	ATCC
P388AD.4	Dr. D. Cohen, University of Kentucky
$P388D_1$	ATCC
S-S.1 (αSRBC)	ATCC
Chemicals	
chloroquine, sodium metrizoate	Sigma Chemical Co.
OPD	Aldrich Chemical Co.
all others	Fisher Scientific Co.
Radiochemicals	
^{125}I	Edmonton Radiopharmaceutical Center
[methyl-^{3}H]-thymidine	New England Nuclear
Miscellaneous	
agarose	L'Industrie Biologique Francaise
Bacto gelatin	Difco Laboratories
collagenase (CLS III)	Worthington
ficoll, percoll, Sephadex G-25	Pharmacia
guinea pig complement	Flow Laboratories
IL-1 (pure recombinant murine)	Dr. P. Lomedico, Hoffman-La Roche
mitomycin C	Sigma Chemical Co.
nylon wool	Fenwall Laboratories
SRBC	Morse Biological Supplies

overnight. After removal of the Sephadex by centrifugation, the supernatant was sterilized by filtration (0.2 μm filter) and stored at -70° C in 10 ml aliquots.

Cell Lines. The DC-like line P388AD.4 and the MØ-like lines P388D$_1$ and J774A-1 were grown as monolayers in RPMI 1640-10% FBS. To harvest the adherent cells, the medium was removed and enough 0.02% (w/v) disodium ethylenediaminetetracetic acid (EDTA) was added to cover the bottom of the culture vessel. After 10 min at room temperature, the cells were dislodged by squirting with a pipette and washed 3 times in RPMI-5% FBS before use. To block cell proliferation for assessment of APC function, P388AD.4 and P388D$_1$ cells (5–10 x 10^6/ml in RPMI-5% FBS) were pretreated with mitomycin C (25 μg/ml for 45 min at 37° C) and washed three times before use. The IL-2 sensitive cell line NKD11 was maintained in RPMI-10% FBS containing 5 x 10^{-5} M 2-ME and an optimal dilution of partially purified IL-2 (see *Interleukin-2 assay* for preparation of IL-2).

Preparation of cell suspensions from lymphoid organs. Mice were killed by cervical dislocation and the lymphoid tissues required (spleen or popliteal lymph nodes) removed. Cell suspensions were obtained by finely mincing the tissue on a sterile stainless steel sieve resting in a dish of Puck's saline. Large debris was removed by sedimentation at 1 *g* for 5 min, after which the cells were centrifuged at 400 *g* for 7 min and resuspended in the appropriate medium for further manipulations. Cell counts were performed using the modified Neubauer hemocytometer and viability was determined using the eosin dye exclusion test (Hanks and Wallace, 1958).

Purification of splenic MØ and DC. Splenic APC were enriched by irradiation and adherence, and were separated into DC-rich and MØ-rich fractions by a modification of several published methods (Steinman *et al*, 1979; Inaba *et al*, 1981; Klinkert *et al*, 1982), as depicted in Figure 1. Forty to fifty spleens were finely minced on a stainless steel sieve, and the loose cells were collected. To recover more MØ, the small fragments retained by the sieve were incubated in 20 ml of Medium 199 (37° C, 30 min, 2 spleen equivalents/ml) in the presence of collagenase (CLS III, 1 mg/ml). The fragments were then mashed on a stainless steel sieve and the cells were pooled with those recovered before collagenase treatment. After

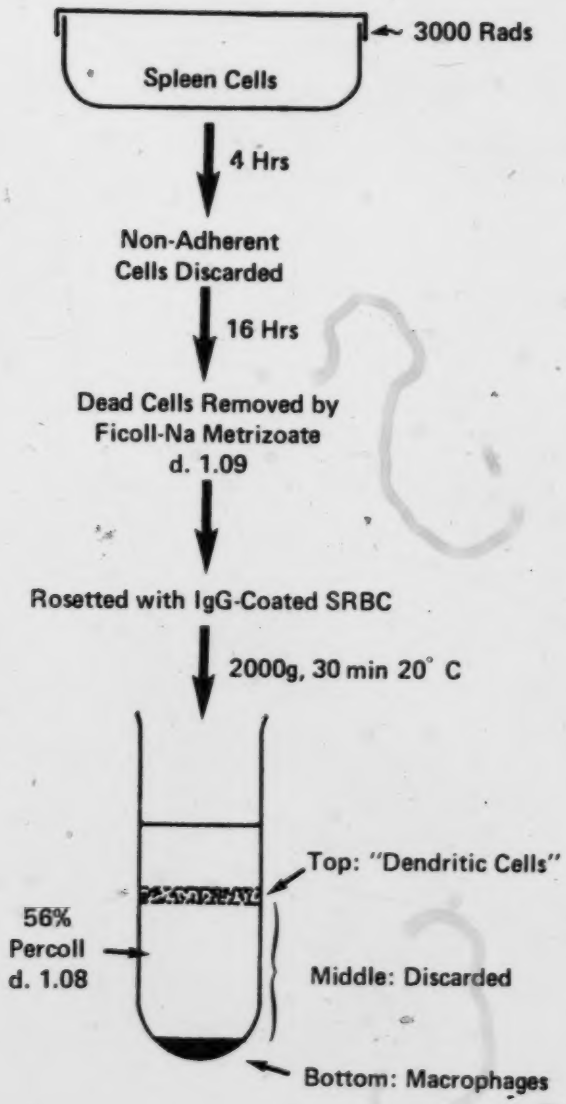

Figure 1. Protocol for purification of splenic DC and MØ.

exposure to 2000 rad γ-irradiation (from a 'Gamma Cell 40', Atomic Energy of Canada Ltd., Ottawa, Ont.,), the cells were incubated for 4 hr in 100 x 20 mm Falcon plastic tissue culture dishes (10^6 cells in 5 ml of RPMI-10% FBS per dish). Nonadherent cells were suspended by rocking 8-10 times and aspirated off. After overnight (18 hr) incubation, the DC and some MØ detached spontaneously, and vigorous pipetting dislodged most adherent MØ. To remove dead cells, the recovered cells (no more than 5 x 10^6/ml) were suspended in 10 ml of RPMI-5% FBS and centrifuged (2000 *g*, 25 min, 20° C) through 15 ml of Ficoll-sodium metrizoate (density = 1.09 g/cm^3), in a 50 ml polystyrene centrifuge tube, according to Parish *et al* (1974). The viable cells were mixed with 100 times their number of IgG-coated SRBC (see below) in serum-free RPMI and incubated for 30 min in ice (Erb *et al*, 1980). To separate rosetted MØ from non-rosetted DC, 10 ml aliquots of the cell suspension were layered over 15 ml of 56.05% percoll (density = 1.08 g/cm^3) in 50 ml polystyrene centrifuge tubes and centrifuged (20° C, 2000 *g*, 30 min). This resulted in a top fraction (1 to 2% of initial spleen cells) containing 70 to 80% DC, 20 to 30% lymphocytes and granulocytes, < 1% Fc rosettes, and a bottom fraction (0.3 to 0.7% of initial spleen cells) predominantly consisting of MØ (98% Fc rosettes). The cells in the percoll between the fractions were discarded.

To prepare IgG-coated SRBC for rosetting with splenic adherent cells, 1 ml of washed, packed SRBC was added to 19 ml of PBS containing Bacto anti-sheep hemolysin at the highest concentration (usually 1/100) that did not agglutinate the SRBC. After 30 min in ice followed by 30 min at 37° C, the SRBC were washed 3 times with PBS and stored in PBS until use on the following day.

Cultivation, fractination, and activation of BM-MØ. The protocol is depicted in Figure 2. BM-MØ were grown from bone marrow cells in the presence of L cell-conditioned medium (LCM) as a source of CSF-1 (Lee and Wong, 1980). This was prepared by incubating mouse L cells (clone L-929) in DMEM-10% FBS (10^6 cells in 20 ml per 75 cm^2 tissue culture flask) at 37° C for 5 days. The CSF-1-containing supernatant was harvested, sterilized by filtration (0.2 μm filter), and stored at -70° C in 40 ml aliquots. Bone marrow cells were flushed from the femur and tibia into Puck's saline and suspended with a syringe and needle.

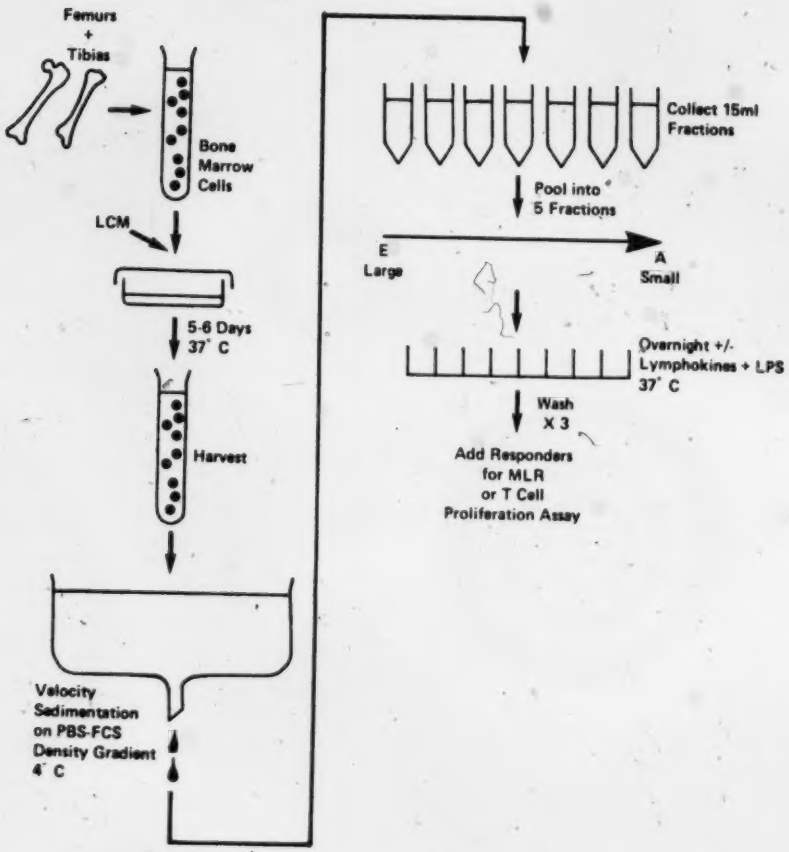

Figure 2. Protocol for growth, size-fractionation, and activation of culture-derived BM-MØ.

After removal of large debris by sedimentation at 1 *g* for 5 min and washing in DMEM-5% FBS, 8 x 10^5 nucleated bone marrow cells were added to each 100 x 20 mm Petri dish (not treated for cell attachment) in 25 ml of DMEM containing 10% LCM, 18% horse serum and 2% FBS. The adherent MØ were harvested after six days. Although the MØ could be easily dislodged by squirting with a Pasteur pipette, they were harvested in Ca^{2+} and Mg^{2+}-free PBS containing 0.02% (w/v) disodium EDTA to minimize clumping during cell fractionation.

To separate functionally distinct MØ subpopulations, MØ were fractionated according to size by the velocity sedimentation technique of Miller and Phillips (1969), as modified for MØ (Lee and Berry, 1977). Briefly, the cells were allowed to sediment at 1 *g* through a gradient of FBS, which served to stabilize the layers of separted cells. Sedimentation velocity is proportional to the square of the cell radius and was calculated as described by Miller (1976). Usually, 75-80% of the MØ were recovered in 40 fractions and were pooled into five fractions (A to E) for experiments. For activation, the MØ were incubated at 37° C in DMEM-10% FBS containing 3 μg/ml *Salmonella typhosa* LPS plus 10% Ia-inducing lymphokines. After 20 hr, the medium was removed and the cells were washed three times *in situ* with warm medium. Antigen and responding T cells were added as indicated in the figure and table legends.

Determination of Ia⁺ cells. Three different methods were employed.

1) Antibody-mediated cytotoxicity. Cell suspensions (5 x 10^6/ml) were incubated for 1 hr in ice (0° C) in Medium 199 containing 0.1% (w/v) Bacto gelatin and an appropriate dilution of 10-2.16 (anti-I-A^k) or MKD6 (anti-I-A^d, specificity control) ascitic fluid. The cells were then washed once and incubated for 45 min at 37° C in the same medium containing guinea pig complement diluted six times. Immediately before use, guinea pig complement was absorbed with agarose (80 mg/ml) for 1 hr at 4° C with constant stirring. The percentage of live cells was determined by eosin dye exclusion.

2) Enzyme-linked immunosorbent assay (ELISA). To reduce nonspecific background binding, BM-MØ monolayers (activated or unactivated) in 96-well flat-bottomed tissue culture trays were preincubated with 0.2 ml of Hank's balanced salt solution (HBSS) containing 0.2% (w/v) Bacto gelatin at 4° C for 1 hr. The medium was then replaced with 0.1 ml of HBSS

containing 0.1% (w/v) Bacto gelatin and an optimal dilution of ascitic fluid or serum-free supernatant from hybridoma cell lines secreting anti-I-A^k, anti-I-A^d or other control monoclonal antibodies (anti-SRBC). This was followed by 0.1 ml of affinity-purified peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$ diluted 300 times in HBSS-0.1% gelatin. Both incubations were for 45 min at 4° C with 3 washes after each incubation. A fresh 5 mg/ml (in methanol) stock solution of the peroxidase substrate o-phenylenediamine (OPD) was diluted 100 times in phosphate-citric acid buffer (0.1 M, pH 5.0) containing 0.003% H_2O_2 (added just before use) and 0.1 ml added to each well. After 30 min incubation at room temperature in the dark, the reaction was stopped with 0.025 ml of 8 N H_2SO_4. The absorbance of each well at 492 nm was read immediately with a Titertek Multiskan plate reader.

3) Flow cytometry. Cells were treated with monoclonal antibodies (1/100 dilution of serum-free supernatants from the indicated hybridomas) essentially as described for the ELISA technique, except that V-bottomed microtiter wells were used and the HBSS contained 0.02% (w/v) sodium azide for all steps. The second antibody was affinity purified fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG $F(ab')_2$ (SAMIG) (1/50 final dilution). Following the last wash, the cells were fixed in 2% (v/v) formaldehyde-PBS and analyzed immediately or on the next day with an EPICS V fluorescence-activated cell sorter (FACS) (Coulter Electronics, Hialeah, FL) using an argon laser exciting at 488 nm, a 492-502 nm blocking filter, and a 525 nm bandpass filter. Fluorescence (log amplification) data were collected at a constant gain setting with the gates for forward angle light scatter adjusted to exclude dead cells and debris. Data were displayed on a histogram with a 255 channel abcissa. On this scale, an increase of 25.6 channels represents a doubling of fluorescence intensity. Relative fluorescence intensities were calculated according to the formula $2^{[positive - negative/25.6]}$, where positive and negative refer to the mean fluorescence intensities of the positive and negative (or specificity) control populations, respectively (Weeks *et al*, 1984). Forward angle light scatter and log 90° C (L90) light scatter of each cell type were also collected. All data were analyzed on the Coulter MDADS computer.

Assessment of APC function. The antigen-presenting activity of various A cell populations was assessed by their ability to induce antigen-specific proliferation in antigen-primed, nylon wool-purified lymph node (LN) T cells or antigen-specific T cell lines. Both sources of T cells required exogenous A cells to proliferate in response to antigen, which was either bound to antigen-pulsed A cells, or added to A cells and T cells at a predetermined optimal concentration. For pulsing with antigen, A cells (not more than 1.5 x 10^7/ml) in RPMI-5% FBS were incubated in 17 x 100 mm tubes with antigen (70 μg/ml for CP and BCG, 100 μg/ml for soluble antigens) for 2 hr at 37° C. The cells were then washed three times to remove antigen before use. Unless otherwise stated, chloroquine (0.3 mM) was added 20 min before the addition of antigen and was removed together with antigen by washing after pulsing (Lee *et al*, 1982).

Nylon wool purification of T cells. Before use, the nylon wool was autoclaved with 0.1 N HCl and then washed by autoclaving 6 times with double distilled water, after which it was thoroughly air dried for 1 wk. Each nylon wool column was prepared by packing 2.5 g of dry nylon wool into the barrel of a 20 ml disposable syringe and autoclaving for sterility.

LN cells were nylon wool filtered by a modification of the technique described by Julius *et al* (1973). The packed column was filled with warm (37° C) RPMI-5% FBS and allowed to drain while long, sterile forceps were used to squeeze out air bubbles. Once the medium reached the top of the nylon wool, LN cells (no more than 2.5 x 10^8 in 4 ml warm medium) were slowly added to the top of the column. Once the cells had entered the column, 2 ml warm medium was added and allowed to enter the column. After 45-60 min incubation at 37° C, the column was washed with 40-50 ml warm medium and the T cell-enriched effluent collected.

Antigen-specific T cell lines. T cell lines were generated by the protocol of Kimoto and Fathman (1980) as shown in Figure 3. Mice were immunized in the hind footpads with an emulsion containing equal proportions of soluble antigen in saline and Complete Freund's Adjuvant H37Ra (CFA) (10 μg antigen in 50 μl/foot). For CP (diluted 10 times in saline), incomplete Freund's Adjuvant (IFA) was used. Popliteal LN were removed after 7 to 9 days and single cell suspensions were passed over nylon wool to obtain a T lymphocyte-enriched, A

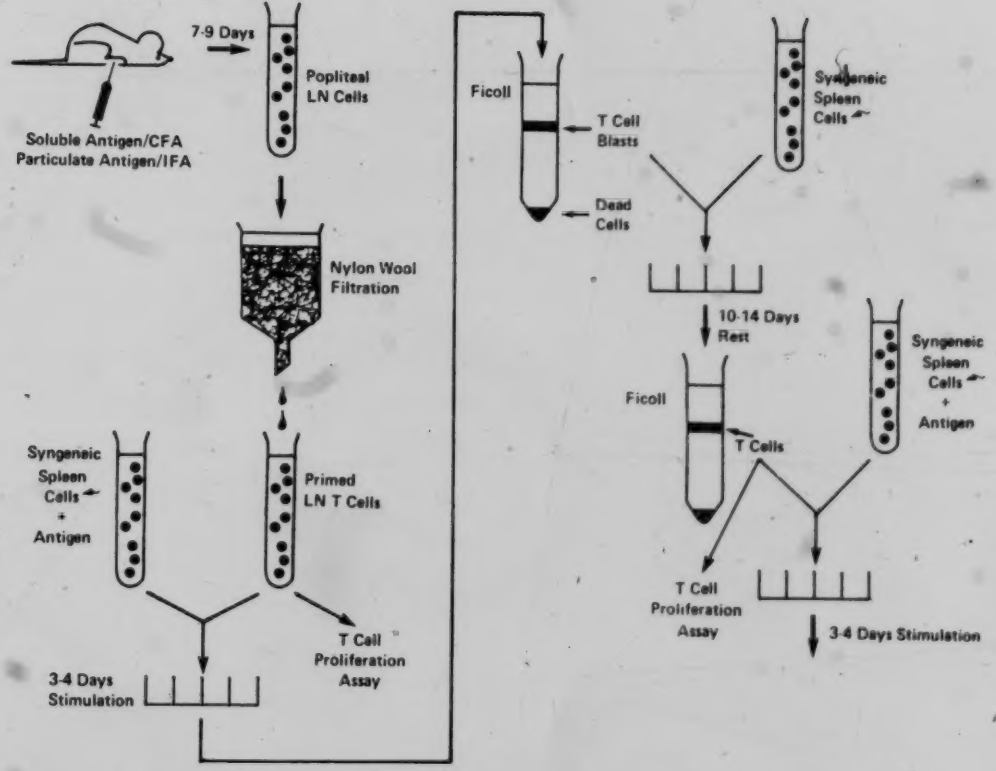

Figure 3. Protocol for establishment of antigen-specific T cell lines. The method is based on Kimoto and Fathman (1980) and is described in detail in *Materials and Methods*.

cell- and B cell-depleted population. T cells (2 x 10^6/ml) were then cultured (in RPMI-10% FBS with 5 x 10^{-5} M 2-ME) with irradiated (2000 rad) syngeneic spleen cells (10^6/ml) and antigen (100 μg/ml for soluble antigens or 70 μg/ml for CP and BCG) in Linbro 24-well tissue culture trays (1 ml/well) at 37° C in a 10% CO_2/air humidified atmosphere. After 3 or 4 days cells were harvested, washed, layered over Ficoll-sodium metrizoate (density=1.09), and centrifuged (800 *g*, 25 min, 20° C). Blast cells were recovered at the ficoll-medium interface, washed, and recultured (10^5/ml, 37° C) with irradiated syngeneic spleen cells (2.5 x 10^6/ml) without antigen in Linbro 24-well tissue culture trays (2 ml/well). After 10-14 days, cells were harvested and recultured (following removal of dead cells by centrifugation through Ficoll-sodium metrizoate) under the same conditions as above, except that antigen was again included. This cycle of antigenic stimulation (for 3-4 days) and resting periods without antigen (10-14 days) was continued indefinitely. Usually, cells were used for experiments after the rest period.

Proliferative responses. Antigen-specific T cell proliferation was measured as the uptake of tritiated thymidine given on the third or fourth day of culture (Figure 4). Nylon wool-filtered primed LN T cells (4 x 10^5/culture) or cells from a T cell line (10^4/culture) were cultured with antigen-pulsed APC or APC plus free antigen (Figure 4). For MLR responses, unfrationated or adherence-depleted spleen cell responders (5 x 10^5/culture) were used and tritiated thymidine was given on the fourth day of culture. For T cell proliferation assays in which nylon wool-purified T cells were used as responders and MLR, RPMI containing 10% human serum was used, since FBS gave high backgrounds. For assays using T cell lines, RPMI-10% FBS and 5 x 10^{-5} M 2-ME was used. All tissue culture media contained 50 μg/ml gentimicin sulphate and 50 IU/ml penicillin-streptomycin.

Six to eight hr before harvesting, 0.6 μCi of [methyl-^{3}H] thymidine (specific activity 20 Ci/mmol) in 25 μl RPMI was added to each well. In some experiments, tritiated thymidine of lower specific activity (2 Ci/mmol) was used and was added 10-18 hr before harvesting. Cells were harvested onto Titertek filter paper with a Titertek cell harvester (Flow Laboratories, Inglewood, CA) and the radioactivity was determined by liquid scintillation

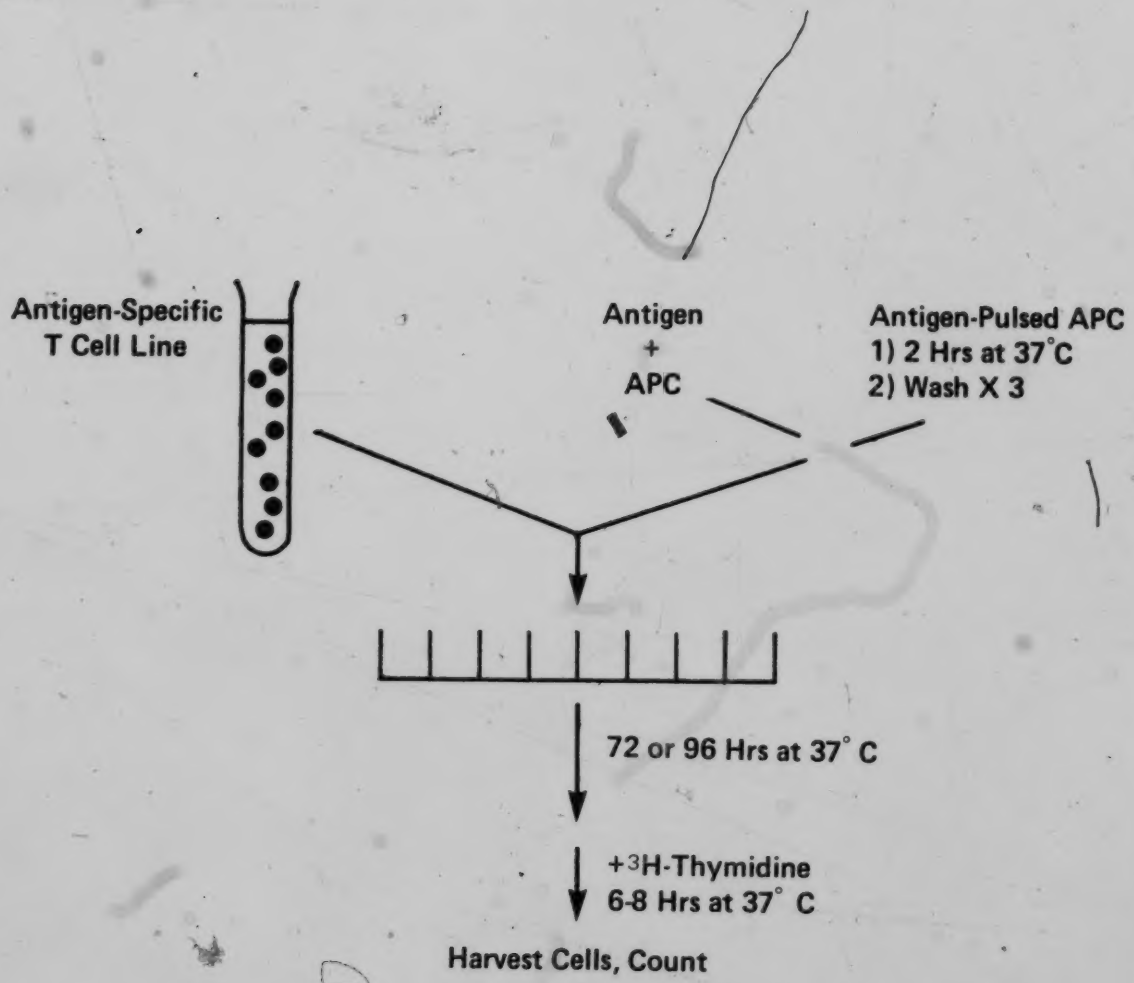

Figure 4. Protocol for T cell proliferation assay. The method is based on Lee *et al*, (1979a) and is fully described in *Materials and Methods*.

spectrometry with an LKB 1218 Rackbeta Counter (Wallac). The results are expressed as mean cpm ± standard deviation (SD) of triplicate cultures. In some cases, proliferative responses to APC without antigen were subtracted and the results expressed as Δcpm.

Interleukin-1 assay. The activity of pure, recombinant IL-1 (Lomedico *et al*, 1984) was assessed by a thymocyte proliferation assay (Lee *et al*, 1981). Briefly, thymocytes (5×10^4/well) from 4-6 wk old C3H/HeJ mice were cultured with or without 6 μg/ml Con A and with various concentrations of IL-1 in RPMI 1640-10% FBS and 5×10^{-5} M 2-ME in 96-well round-bottomed trays. Tritiated thymidine uptake was determined on day 3.

Interleukin-2 assay. For IL-2 assays, IL-2-dependent NKD11 cells (10^4/culture) were cultured in medium only, or with serial dilutions of supernatants from antigen-activated T cell lines. An optimal dilution of partially purified IL-2 was included as a positive control. The uptake of tritiated thymidine was determined on the second day.

IL-2 was partially purified from the supernatant of the EL-4 thymoma cell line. To increase IL-2 production, EL-4 cells ($0.5 - 1 \times 10^6$/ml in RPMI with 5% v/v horse serum) were cultured with phorbol myristate acetate (10 ng/ml) for 18-24 hr prior to harvesting the supernatant. The IL-2 was concentrated by precipitating with 51.6 g ammonium sulphate per 100 ml supernatant. After 2 hr with stirring, the IL-2-containing precipitate was pelleted by centrifugation at 10,000 rpm for 15 min (GSA rotor, Sorval RC-3), dissolved in a minimum amount of PBS, and dialyzed exhaustively against PBS. After removal of any remaining precipitate by centrifugation (800 *g*, 20 min), the solution was sterilized by filtration (0.2 μm filter) and stored at -70° C.

III. RESULTS

A. Purification of DC and MØ from Mouse Spleen

Initially, several published methods for the purification of DC and MØ from mouse spleen were tried. The original Steinman method, which involves first separating low density spleen cells (primarily MØ and DC) from high density lymphocytes using bovine serum albumin gradients, was difficult to work with and did not result in MØ- and DC -enriched fractions of acceptable purity. Inaba *et al* (1981) cultured splenic adherent cells overnight, collected those cells which detached, and subjected them to Fc-rosetting to separate MØ from DC. This procedure resulted in only 20-30% DC in the non-rosette fraction, which was not considered sufficiently pure for the studies to be undertaken in this thesis. Klinkert *et al* (1980,1982) adapted the Steinman technique for the purification of rat DC, which are nonadherent, by irradiating the cells prior to overnight culture so that most of the non-DC nonadherent cells (mostly lymphocytes) die during the culture period and can be selectively removed the next day by centrifugation through Ficoll-metrizoate. Thus, the final procedure (described in *Materials and Methods* and depicted in Figure 1) adopted for these studies is a modification of several published techniques and was found to be surperior from the standpoint of ease of execution and purity of the cell fractions obtained.

The composition of the MØ and DC fractions following enrichment from murine splenic adherent cells is shown in Table 3. The MØ fraction was 98% FcR^+ MØ by morphology, but not all of these were phagocytic for polystyrene beads, since splenic MØ are immature (Steinman *et al*, 1979) and are less phagocytic than mature MØ (Lee and Wong, 1982). Although the DC fraction was less pure, the contaminating cell types were granulocytes, lymphocytes, and stromal cells, and MØ contaminants were undetected ($< 1\%$). This level of contamination with non-DC and non-MØ cell types is not uncommon (Naito *et al*, 1984) and should not be critical, since a range of cell concentrations was always tested for a particular APC function. The important thing is that MØ cross-contamination was minimal. The MØ and DC fractions contained similar numbers (60-70%) of Ia^+ cells, as determined by

TABLE 3

Composition of MØ and DC Fractions Following Enrichment from Splenic Adherent Cells

Percoll Fraction[a]	Composition
Top (DC-enriched)	70-80% DC[b]
	20-30% lymphocytes, granulocytes, and stromal cells
	< 1% Fc rosettes
	60-70% Ia^+ (minimum estimate)[c]
Bottom	> 98% Fc rosettes
(MØ-enriched)	60-70% Ia^+ (minimum estimate)[c]

[a] Purification of splenic DC and MØ described in *Materials and Methods*.

[b] Proportion of DC determined morphologically in Giemsa-stained smears.

[c] Determined by αIa plus complement cytotoxicity.

complement-mediated cytotoxicity using anti-I-A^k antibody (10-2.16), or anti-I-A^d antibody (MKD6) as a specificity control.

B. Growth and Characterization of Culture-Derived BM-MØ

Pure MØ were cultivated from small numbers of bone marrow cells grown with L cell-conditioned medium (LCM) as a source of MØ growth factor (Lee and Wong, 1980). The growth curves were published previously (Lee and Wong, 1980) and will not be presented here. I have confirmed that adherent cells harvested during late logarithmic phase (five or six days of culture) are 100% MØ based on morphology, phagocytic activity, FcR expression, and staining for enzymes such as esterase amd β-glucuronidase (Lee and Wong, 1980). After 6 days of growth, MØ of various maturational stages and sizes are present. The maturation of MØ is associated with an increase in cell size, since myeloperoxidase, an enzyme marker for immature MØ, is concentrated in the small MØ (Lee and Wong, 1982). Since BM-MØ display functional heterogeneity according to the maturational state (and therefore size) (Lee, 1980a; Lee and Wong, 1980,1982), BM-MØ were always fractionated by size before assessment of APC function. Flow cytometry analysis of cell size (represented by forward angle light scatter profiles) confirmed the effectiveness of the velocity sedimentation technique for fractionation of cells according to size (Figure 5). Thus, the mean light scatter (channel no.) for fraction A (slowest sedimentation velocity) was 75 ± 27 *vs* 119 ± 40 for fraction E (fastest sedimentation velocity).

Previous work has shown that BM-MØ grown under these conditions contain few Ia$^+$ cells if they have not been activated with lymphokines (Calami *et al*, 1982; Lee and Wong, 1982). Therefore, before assessment of APC function, BM-MØ were incubated overnight with Ia-inducing lymphokines (supernatant fluids from Con A-activated rat spleen cells) and LPS. These activation conditions were based on previous results showing that they produce maximal activation and functional discrimination between small immunostimulatory MØ and large suppressive and tumoricidal MØ (Lee and Wong, 1982). Activation was performed on irradiated BM-MØ without LCM since the growth stimulus of CSF-1 inhibits Ia induction by T

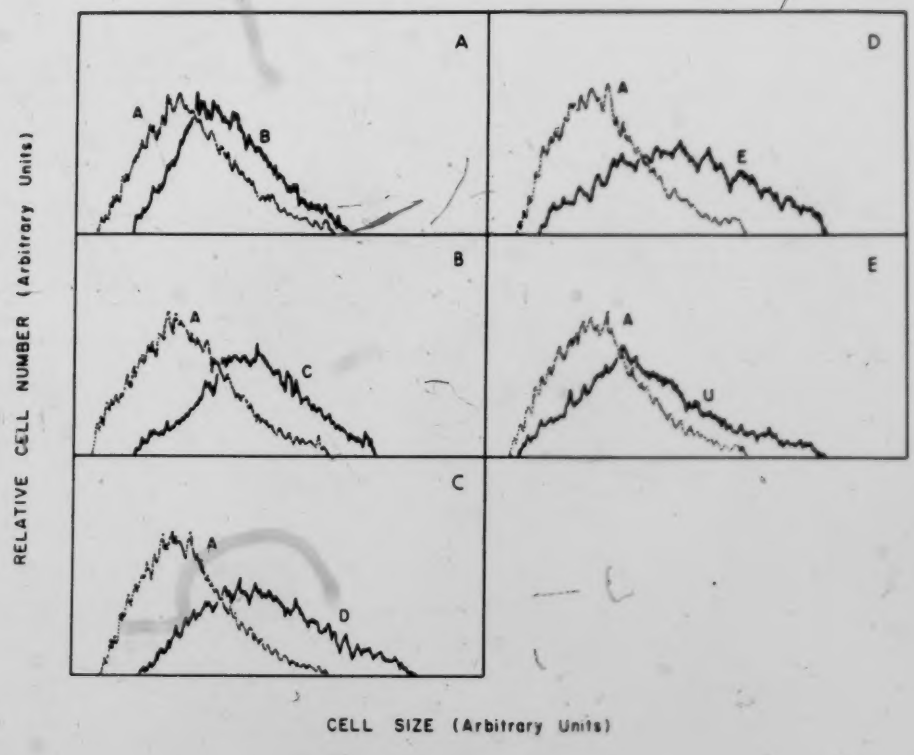

Figure 5. Size comparison of BM-MØ fractions by flow cytometry. After 6 days of growth LCM, BM-MØ were harvested and fractionated according to size by velocity sedimentation. Forward angle light scatter flow cytometry profiles (linear scale) of fractions A-E and unfractionated (U) BM-MØ were collected. Gates were set to exclude dead cells and debris from analysis. The mean light scatter (channel no.) ± SD of the fractions were: A, 75 ± 27; B, 93 ± 27; C, 114 ± 30; D, 115 ± 40; E, 119 ± 40; and U, 99 ± 41. Sedimentation velocities in mm/hr were: A, 2.0-5.8; B, 5.8-7.6; C, 7.6-9.1; D, 9.1-10.7; and E, 10.7-12.9. The percentages of cells recovered in fractions A to E were: 11.1, 34.5, 22.9, 21.2, and 10.3.

cell factors (Calami *et al*, 1982). To confirm that activation was necessary for Ia induction in the majority of BM-MØ, an ELISA assay was performed on normal and activated unfractionated BM-MØ treated with monoclonal anti-I-A^k or anti-I-A^d antibody, followed by peroxidase-conjugated goat anti-mouse IgG (Table 4). The results showed that the low levels of I-A^k and I-A^d expressed by unactivated CBA/CaJ and DBA/2J BM-MØ respectively, were increased several-fold following overnight activation with lymphokines plus LPS. Although the anti-I-A^k and anti-I-A^d antibodies were of different isotypes and therefore differential nonspecific binding to distinct FcR (Unkeless, 1980) could have been problematic, nonspecific binding to FcR was not observed at the concentrations used. Thus, binding to unactivated MØ (mostly Ia^-, but FcR^+) of the appropriate haplotype was much lower than the positive result, and binding to any MØ of the wrong haplotype was indistinguishable from that obtained without antibody, or with irrelevant anti-SRBC monoclonal antibodies of several isotypes (eg., N-S.8.1, IgG_{2b}; S-S1, IgG_{2a}). Similar results were obtained by Beller *et al* (1980), who used the same antibody (10-2.16) to detect I-A^k on MØ. More detailed analysis of Ia expression using the FACS will be presented in a later section.

C. MLR Stimulation

Inasmuch as the allogeneic MLR is an *in vitro* correlate of the allograft response, identification of the stimulator cell(s) involved is central to the design of strategies for the prevention or reduction of host immune reactions against allogeneic tissue. Early studies in the human (Rode and Gordon, 1974), guinea pig (Greineder and Rosenthal, 1975) and murine (Talmage and Hemmingsen, 1975) systems attributed this stimulatory function to MØ, however, the cell purification techniques used were crude and probably did not separate DC from MØ in the adherent cell populations. More recently, splenic MØ were shown to be effective MLR stimulators (Minami *et al*, 1980; Sunshine *et al*, 1982), but Steinman *et al* (1983) argued that this activity could have been entirely due to low numbers of contaminating DC, in view of their potency in this regard. In an attempt to resolve this issue, two highly purified MØ subpopulations were compared to DC as stimulators for allogeneic MLR.

TABLE 4

ELISA Assay for Ia Expression on Unactivated and Activated BM-MØ

BM-MØ		Absorbance at 492 nm (mean ± SD/culture)[a]			
		Antibody[b]			
Strain	Activation[c]	10-2.16	MKD6	N-S.8.1	S-S.1
A) CBA/CaJ	-	0.08 ± 0.02	< 0.02	ND[d]	ND
	+	0.37 ± 0.06	< 0.02	ND	ND
B) DBA/2J	-	< 0.005	0.01	< 0.005	< 0.005
	+	< 0.005	0.12 ± 0.01	< 0.005	< 0.005

[a] Absorbance of BM-MØ monolayers at 492 nm was determined in quadruplicate following the ELISA protocol described in *Materials and Methods*.

[b] For experiment A, serum-free supernatants from the 10-2.16 (anti-I-A^k-specific) and MKD6 (anti-I-A^d-specific) hybridomas were used at a final dilution of 1/100. For experiment B, ascitic fluids from each hybridoma were used at a final dilution of 1/1500. The absorbance of MØ treated with peroxidase-conjugated anti-mouse IgG alone was < 0.02 (experiment A) and < 0.005 (experiment B). SD < 0.01 are not shown.

[c] 8.5×10^4/well unfractionated CBA/CaJ (experiment A) or 10^5/well DBA/2J (experiment B) BM-MØ were cultured overnight in DMEM-10% FBS with or without 10% Ia-inducing lymphokines and 3 μg/ml LPS. MØ monolayers were washed 3 times with warm medium prior to the ELISA assay.

[d] ND, not determined.

Splenic MØ and DC as Stimulators for MLR

Initially, MØ and DC purified from mouse spleen were compared as stimulators for allogeneic and syngeneic MLR. Results from a typical experiment are depicted in Figure 6. The mouse strains chosen were identical at the mixed lymphocyte-stimulating (*Mls*) locus (see Table 1), and hence recognition of self and allogeneic Ia probably accounted for most of the MLR. In agreement with previous reports (Nussenzweig and Steinman, 1980; Kuntz Crow and Kunkel, 1982; Van Voorhis *et al*, 1983), the most striking difference between the two cell types lies in the stimulation of syngeneic MLR; only DC had any detectable activity.

Because syngeneic MLR stimulation was obtained with DC only at high cell concentrations, at which MØ could have been suppressive, a cell-mixing experiment was performed to test the possibility that the failure of MØ to induce syngeneic MLR was due to nonspecific suppression. No evidence of this was found (Table 5). Thus, the proliferative responses induced by DC mixed with equal numbers of non-stimulatory MØ were similar to DC alone.

For allogeneic MLR stimulation, DC and MØ had comparable activities, and their dose-response curves and optimal cell concentrations were similar (Figure 6). These findings suggest that the activity in each fraction was not due to cross-contamination by small numbers of the other cell type. However, the possibility that small numbers of contaminating DC contributed to the magnitude of the response induced by MØ could not be ruled out. Therefore, it was necessary to show that a pure population of MØ could stimulate MLR. To this end, culture-derived BM-MØ (100% pure), were used. Since the suppressive activity of large MØ could obscure the stimulatory activity of the small ones, the MØ were fractionated according to size before use.

BM-MØ as Stimulators for MLR

In accordance with their low level of Ia expression, unactivated BM-MØ were inactive at initiating allogeneic MLR at all concentrations tested ($3 \times 10^3 - 10^5$/culture), although only the results for one concentration are shown (Figure 7). Activated BM-MØ stimulated good

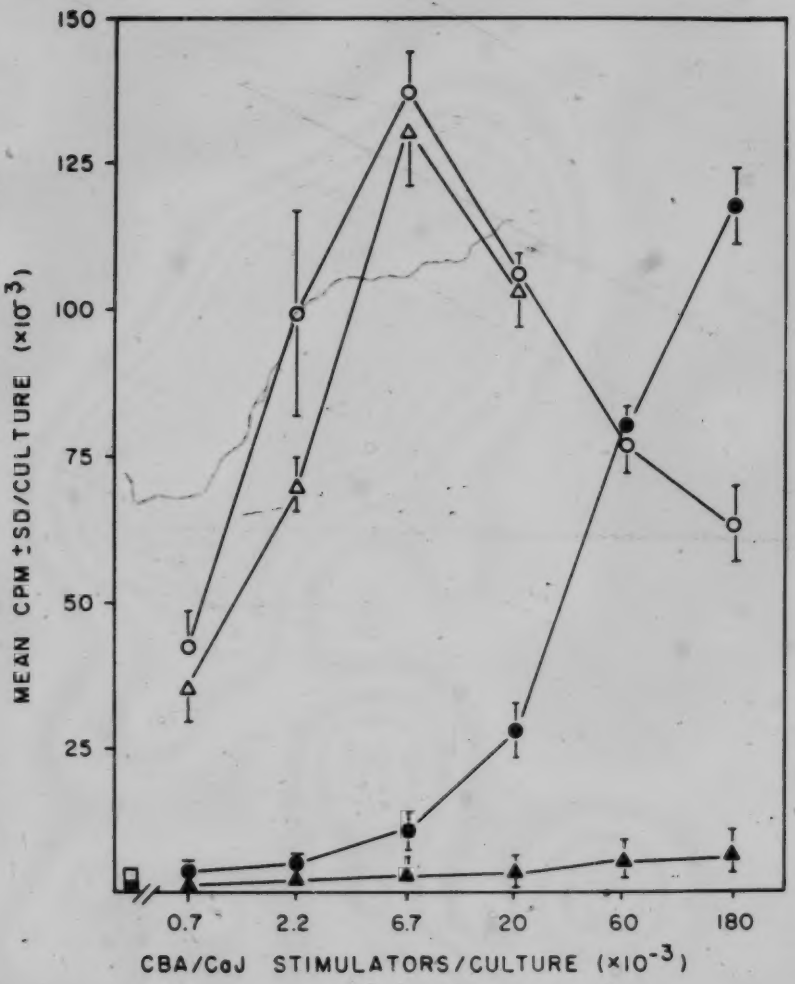

Figure 6. MLR stimulation by CBA/CaJ splenic MØ and DC. Syngeneic CBA/CaJ (closed symbols) or allogeneic BALB/cCR (open symbols) spleen cell responders (5×10^5/culture) were cultured alone (squares) or with the indicated numbers of CBA/CaJ splenic MØ (triangles) or DC (circles).

TABLE 5

Lack of MØ-Induced Suppression of Syngeneic MLR Activation by DC

Stimulating Cells		Proliferative Response[a] (mean cpm ± SD/culture ($\times 10^{-3}$))
Type	No./Culture ($\times 10^{-3}$)	
	0	3
DC	6.7	7 ± 1
	22	26 ± 8
	67	127 ± 4
	200	134 ± 13
MØ	6.7	3 ± 1
	22	6
	67	4 ± 1
	200	4 ± 1
DC + MØ	3.3 + 3.3	8
(1:1)	11 + 11	28 ± 3
	34 + 34	74 ± 4
	100 + 100	89 ± 2

[a] Syngeneic CBA/CaJ responder spleen cells, 5×10^5/culture. SD < 1000 cpm are not shown.

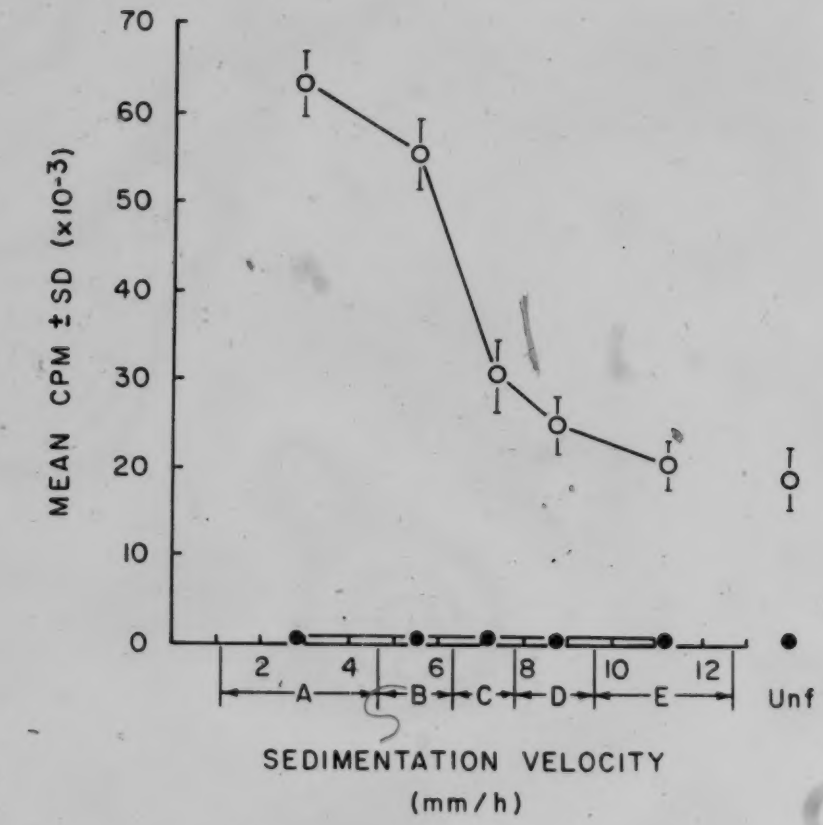

Figure 7. MLR stimulation by activated and unactivated BM-MØ. The allogeneic MLR stimulating capacity of unactivated (closed circles) or activated (open circles) CBA/CaJ BM-MØ (10^5/culture) was compared. BALB/cCR nonadherent spleen cell responders (5×10^5/culture) were used. Responses without APC were < 300 cpm. Prior to activation, BM-MØ were fractionated according to size by velocity sedimentation (A-E) or left unfractionated (U). Sedimentation velocities of BM-MØ fractions (mm/hr) were: A, 1.1-4.7; B, 4.7-6.4; C, 6.4-7.8; D, 7.8-9.6; and E, 9.6-12.7. The percentages of cells recovered in fractions A to E were: 12.1, 24.4, 27.2, 20.5 and 15.8.

allogeneic MLR, and in agreement with previous results for antigen-specific T cell proliferation (Lee and Wong, 1982), the activity was associated mainly with small BM-MØ. A more detailed assessment of MLR stimulation by activated BM-MØ in comparison with splenic DC and MØ is shown in Figure 8. The smallest (slowest sedimenting) activated BM-MØ in fractions A and B were the best MØ stimulators of allogeneic MLR and were almost as potent as DC (Figure 8A). Similarly, they consistently stimulated the proliferation of syngeneic, APC-depleted T cells, albeit to a much lesser degree than DC (Figure 8B). In contrast, large or unfractionated BM-MØ were poor stimulators of both allogeneic and syngeneic MLR (Figure 8). This could have been due to the suppressive activity of large MØ reported previously (Lee and Wong, 1982). The allogeneic MLR stimulating capacity of splenic MØ was variable and could be comparable to DC as in the earlier studies (Figure 6) or somewhat lower (Figure 8A). In the initial studies, splenic MØ did not elicit syngeneic MLR from unfractionated spleen cell responders (Figure 6); however, the adherent cell-depleted splenic T cell responders used in the later studies responded slightly, but reproducibly, to syngeneic splenic MØ and small activated BM-MØ (Figure 8B). These results demonstrate that MØ free from DC are effective stimulators of allogeneic MLR and, albeit to a lesser extent, syngeneic MLR.

It should be emphasized that the culture conditions for the proliferative responses have been optimized and changes in the shape of culture wells or time of thymidine pulse would not affect the conclusions, although the responses would be quantitatively different. For example, responder cells in round-bottomed wells required fewer APC to attain a given level of stimulation, but the background response (without APC) was higher (data not shown).

D. Presentation of Soluble and Particulate Antigens

In the initial studies comparing DC and MØ as APC for antigen-specific T cell activation, antigen-primed, nylon wool-purified LN T cells were used as responders. However, the potent syngeneic MLR-stimulating capacity of DC resulted in high background responses to DC in the absence of antigen, thus making interpretation difficult. For this reason,

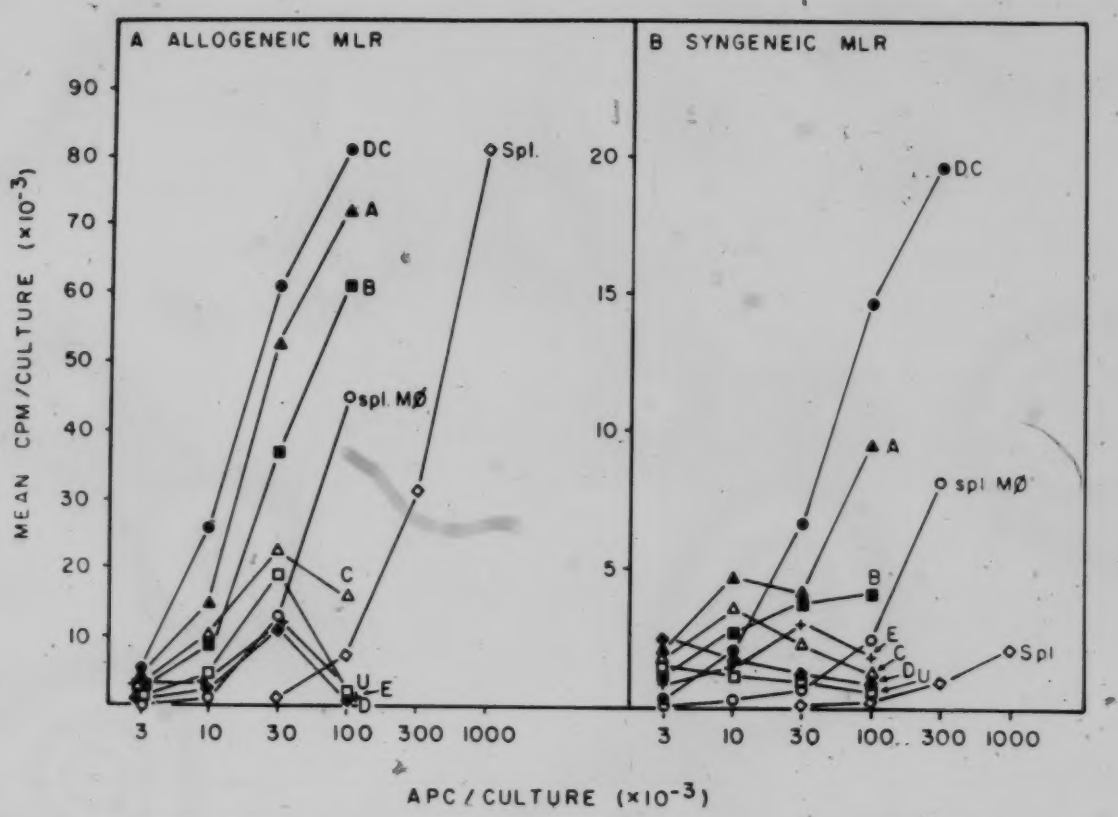

Figure 8. MLR stimulation by splenic MØ, splenic DC, and activated BM-MØ. Allogeneic BALB/cCR (A) or syngeneic CBA/CaJ (B) nonadherent responder spleen cells (5 x 10^5/culture) were cultured with the indicated numbers of CBA/CaJ splenic MØ, DC, or activated, size-fractionated BM-MØ (A-E). Unfractionated (U) BM-MØ were also tested. Responses without APC were < 300 cpm. Standard deviations were < 10% of the mean. Sedimentation velocities of BM-MØ fractions in mm/hr were: A, 2.5-6.0; B, 6.0-7.9; C, 7.9-9.5; D, 9.5-11.1; and E, 11.1-13.3. The percentages of cells recovered in fractions A to E were: 18.9, 22.8, 21.3, 17.1, and 19.9.

antigen-specific T cell lines selected for low background were used as responders for most of the antigen-presentation studies.

Characterization of Antigen-Specific T Cell Lines

The procedure for the establishment of antigen-specific T cell lines, based on Kimoto and Fathman (1980), is fully described in ***Materials and Methods*** and is summarized in Figure 3. The antigen-specificity of each line was routinely tested. Those lines which proliferated to APC alone, or to antigens other than those used for priming, were discarded. Some typical results are shown in Table 6. The time course for antigen-dependent proliferation was also determined for each T cell line because the day on which the proliferation peaked varied with the line, but was usually day 3 or day 4. Representative examples are displayed in Table 7.

To further demonstrate that the proliferative response of the T cell lines was comparable to freshly explanted T cells, the requirement for MHC restriction was assessed. Only spleen cells syngeneic to the T cell line provided significant APC function (Table 8). Moreover, the proliferation of H-2^k T cell lines in the presence of H-2^k APC was significantly blocked by monoclonal anti-I-A^k antibody (Table 9), but not by anti-I-A^d antibody. The incomplete inhibition by anti-I-A^k could have been due to the proliferation of I-E^k-restricted T cells in the population.

Although T cell proliferation is generally considered to reflect helper activity, there have been reports of T cell proliferation in the absence of demonstrable helper functions (Ramila and Erb, 1983; Ramilla *et al*, 1985). Therefore, the functional capabilities of several T cell lines were assessed. Not surprisingly, the lines produced significant levels of IL-2 in an antigen- and APC-dependent manner, and the levels produced by the lines were very similar (Figure 9). In collaboration with Dr. A. Sinha, it was found that a KLH-specific line provided carrier-specific help for unprimed, hapten-specific B cells (Table 10). Furthermore, CP-specific (Tables 11 and 12) and PPD-specific lines delivered antigen-specific, carrier-dependent help to alloreactive CTL precursors, although such help did not depend on

TABLE 6

Antigen-Specificity of T Cell Lines

Line[a]	Antigen[b]	Proliferative Response[c] (mean Δcpm ± SD/culture ($\times 10^{-3}$))
C-PPD	PPD	73 ± 4
	POL	2 ± 1
C-POL	POL	83 ± 10
	PPD	1
C-CP	CP	89 ± 8
	BCG	4 ± 2
C-KLH	KLH	30 ± 5
	PPD	2 ± 1

[a] Background proliferation without APC for CBA/CaJ lines C-PPD, C-POL, C-CP, and C-KLH was 0.2, 0.3, 0.2, and 0.1 cpm ($\times 10^{-3}$)/culture, respectively.

[b] PPD, POL, and KLH were included in culture at 50 μg/ml. CP and BCG were included at 20 μg/ml. Syngeneic irradiated spleen cells (10^6/culture) were included as a source of APC.

[c] Responses to APC without antigen have been subtracted. SD < 1000 cpm are not shown.

TABLE 7

Time Course for Antigen-Specific Proliferation of T Cell Lines

Line[a]	Proliferative Response[b] (mean Δcpm ± SD/culture (x 10^{-3}))		
	day 2	day 3	day 4
C-CP	5 ± 1	8 ± 1	7 ± 1
C-PPD	19 ± 2	43 ± 1	75 ± 4
C-BCG	36 ± 2	99 ± 10	58 ± 2
C-KLH	27 ± 1	87 ± 5	114 ± 7
D-CP	45 ± 4	99 ± 5	115 ± 7
D-PPD	23 ± 1	47 ± 5	79 ± 6
D-BCG	36 ± 1	109 ± 12	124 ± 11

[a] Background proliferation without APC for CBA/CaJ lines C-CP, C-PPD, C-BCG, and C-KLH, and for DBA/2J lines D-CP, D-PPD and D-BCG was 0.1, 0.2, 0.1, 0.3, 0.2, 0.2, and 0.3 cpm (x 10^{-3})/culture, respectively. Concentrations of antigen and APC were the same as described for Table 6.

[b] Proliferative responses were assessed on the indicated days. Responses to APC without antigen have been subtracted.

TABLE 8

MHC Restriction of Antigen-Specific T Cell Lines

Line[a]	Proliferative Response[b] (mean Δcpm ± SD/culture (x 10^{-3}))	
	APC	
	CBA/CaJ	BALB/cCR
C-CP	68 ± 9	3 ± 1
C-KLH	32 ± 3	2 ± 1
C-PPD	37 ± 5	1
B-CP	5 ± 2	85 ± 10
B-PPD	3 ± 1	48 ± 5
B-BCG	4 ± 2	37 ± 4

[a] Background proliferation for CBA/CaJ lines C-CP, C-KLH, and C-PPD and for BALB/cCR lines, B-CP, B-PPD, and B-BCG was 0.3, 0.2, 0.2, 0.3, 0.1, and 0.2, respectively. Concentration of antigen and APC were the same as described for Table 6.

[b] Responses to APC without antigen have been subtracted. SD < 1000 cpm not shown.

TABLE 9

Specific Inhibition of T Cell Proliferation by Monoclonal αI-A^k Antibody

Line[a]	No. APC/Culture[b] ($\times 10^{-4}$)	Proliferative Response[c] (mean Δcpm ± SD/culture ($\times 10^{-3}$))		
		medium	αI-A^k	αI-A^d
C-CP	6.7	12 ± 2	2 ± 1	8 ± 2
	20	34 ± 6	7 ± 6	32 ± 3
	67	82 ± 10	13 ± 2	77 ± 8
	200	142 ± 17	25 ± 3	126 ± 15
C-KLH	3	3 ± 1	ND[d]	ND
	10	9 ± 2	3 ± 1	9 ± 1
	30	16 ± 3	7 ± 1	14 ± 1
	100	17 ± 2	4 ± 1	15 ± 1

[a] Background proliferation without APC for CBA/CaJ lines C-CP and C-KLH was 0.5 and 0.1 cpm ($\times 10^{-3}$)/culture respectively; CP and KLH were included at 20 μg/ml and 25 μg/ml, respectively.

[b] Syngeneic irradiated spleen cells (10^6/culture) were included as a source of APC.

[c] Responses to APC without antigen have been subtracted. Monoclonal αI-A^k or αI-A^d were included in culture as serum-free culture supernatants (1/1000 dilution) of the B cell hybridomas 10-2.16 and MKD6, respectively.

[d] ND, not determined.

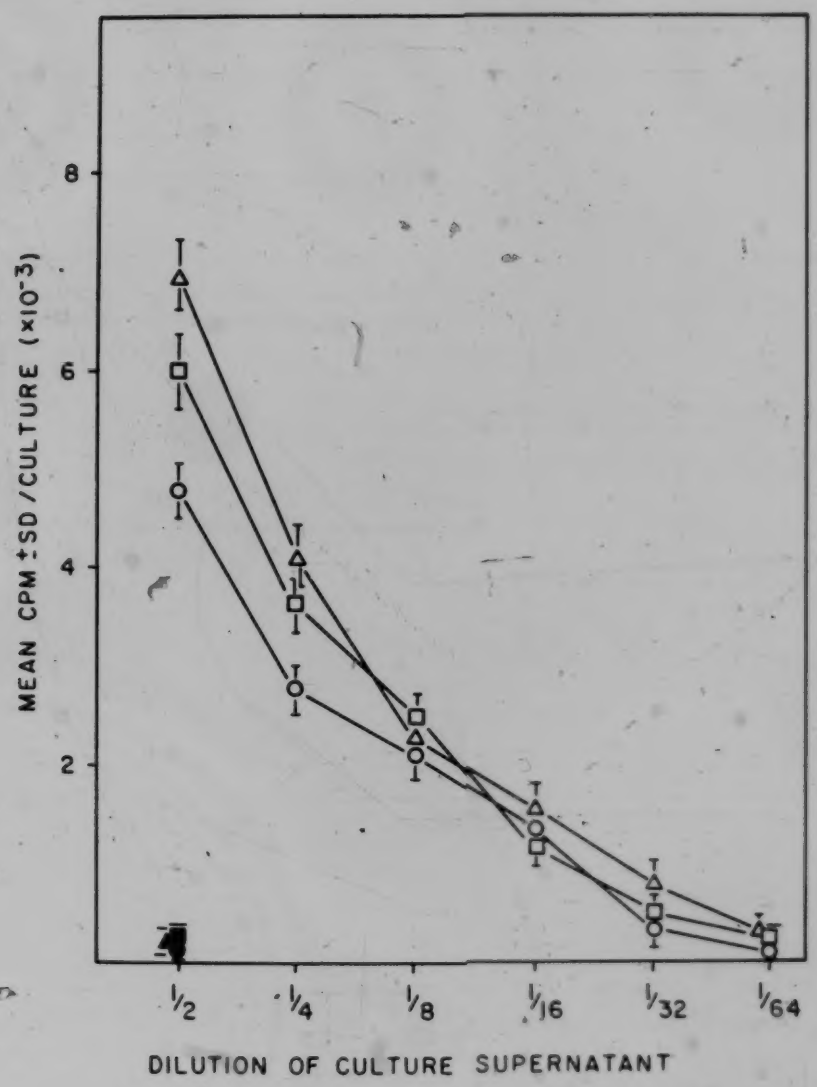

Figure 9. T cell lines release IL-2 in response to antigen and APC. CBA/CaJ T cell lines (10^4/culture) specific for PPD (circles), KLH (squares), or CP (triangles) were cultured with 10^6 CBA/CaJ irradiated spleen cells as a source of APC in the absence (closed symbols) or presence (open symbols) of 25 μg/ml PPD, KLH, or 20 μg/ml CP, respectively. After 24 hr, the indicated dilutions of supernatants were assayed for IL-2 using the IL-2 sensitive line NKD11. The mean cpm ± SD/culture (x 10^{-3}) incorporated by NKD11 cells cultured in control medium (without IL-2) or with an optimal dilution (1/40) of IL-2 containing supernatant from the EL-4 thymoma were 0.2 and 20.8 ± 1, respectively.

TABLE 10

B Cell Helper Activity of C-KLH T Cell Line†

No. T Cells/Culture ($\times 10^{-3}$)	Mean anti-TNP AFC ± SD/10^4 cells[a]	
	Antigen in culture[b]	
	TNP-KLH	TNP-CGG
0	< 10	< 10
1	127 ± 12	16 ± 4
3	319 ± 27	29 ± 6
10	629 ± 21	11 ± 2
30	315 ± 41	11 ± 2

[a] CBA/CaJ spleen cells (10^6/culture) were cultured alone or with the indicated numbers of C-KLH T cells. AFC responses were assessed on day 5 (Diner *et al*, 1986).

[b] TNP-KLH or TNP-CGG were included in culture at 0.1 μg/ml. The AFC response to the T-independent antigen TNP-Ba was 774 ± 41 AFC/10^6 cells. Background AFC responses in cultures without antigen were < 10 AFC/10^6 cells and have been subtracted from all values.

† Done in collaboration with Dr. A. Sinha.

TABLE 11

Carrier-Dependent CTL Helper Activity of the D-CP T Cell Line†

APC[a]	No. T Cells/Culture[b] (x 10^{-2})	IL-2[c]	% Specific Lysis
Unpulsed	0	-	< 1
	0	+	35.8 ± 5.0
	0.1	-	< 1
	1	-	< 1
	10	-	< 1
CP-pulsed	0	-	< 1
(7 μg/ml)	0	+	48.4 ± 8.6
	0.1	-	< 1
	1	-	10.9 ± 1.9
	10	-	21.5 ± 3.6
CP-pulsed	0	-	< 1
(70 μg/ml)	0	+	44.7 ± 4.6
	0.1	-	5.2 ± 2.9
	1	-	6.0 ± 4.7
	10	-	31.7 ± 6.7

[a] (BALB/cCR x CBA/J)F_1 irradiated stimulator cells (3 x 10^5/culture) were pulsed with the indicated CP concentrations for 90 min at 37° C, or left unpulsed, after which 10^5 CBA/J thymocyte responders (from 4-6 wk old mice) were added.

[b] T cells (D-CP T cell line) were irradiated (1500 rad) prior to culture.

[c] Optimal concentration (1/100) of partially purified EL-4 culture supernatant.

[d] Cytotoxicity assay (4 hr ^{51}Cr release) against BALB/cCR Con A-induced blast cells was performed after 5 days of culture (Krowka *et al*, 1984).

† Done in collaboration with Dr. J. Krowka.

TABLE 12

Antigen-Specific CTL Helper Activity of the C-CP T Cell Line†

APC[a]	No. T Cells/Culture[b] ($\times 10^{-2}$)	% Specific Lysis[c]
CP-pulsed	0	< 1
	1	< 1
	3	< 1
	10	1.2 ± 3.4
	30	20.8 ± 3.8
PPD-pulsed	0	< 1
	1	< 1
	3	< 1
	10	< 1
	30	< 1

[a] (BALB/cCR x CBA/J)F_1 irradiated stimulator cells (3×10^5/culture) were pulsed with CP (70 μg/ml) or PPD (50 μg/ml) for 90 min at 37° C, and washed 3 times prior to the addition of 10^5 CBA/J thymocytes (from 4-6 wk old mice) as responders.

[b] T cells (C-CP T cell line) were irradiated (1500 rad) before culture.

[c] Cytotoxicity assay (4 hr ^{51}Cr release) against BALB/cCR Con A induced blast cells was performed after 5 days of culture (Krowka *et al*, 1984). An optimal concentration (1/100) of partially-purified IL-2 from the EL-4 thymoma induced 53.5 ± 6.2 and 43.7 ± 7.2 % specific lysis in the absence of T cells but in the presence of CP-pulsed and PPD-pulsed stimulators, respectively.

† Done in collabaration with Dr. J. Krowka.

the ability of the T cells to proliferate (done in collaboration with Dr. J. Krowka).

These results demonstrate that the T cell lines used in these studies proliferate in an antigen-specific, I region-restricted manner, and deliver antigen-specific help for *in vitro* B cell and CTL activation.

Comparison of Splenic MØ and DC as APC for Soluble and Particulate Antigen Presentation

The existence of separate phagocytic (MØ) and nonphagocytic (DC) APC lineages suggests that functional and cellular diversity is needed to process and present the large variety of antigenic forms in nature. By virtue of their phagocytic and lysosomal activity, MØ may be the main processors of complex antigens, whereas DC, with strong constitutive Ia expression and minimal phagocytic activity (reviewed in Steinman and Nussenzweig, 1980), may be particularly suited for surface presentation of antigens not requiring processing. To test this possibility, splenic MØ and DC were compared for the ability to present soluble antigens (both small and large) and whole bacteria to antigen-specific T cell lines.

MØ at low cell concentrations had similar or slightly lower activity than DC at presenting soluble antigens such as PPD and POL to the corresponding T cell lines (Figure 10). At higher concentrations, the activity of MØ fell significantly, possibly due to nonspecific suppression. On the other hand, MØ were between three to nine times better (on a per cell basis) than DC at presenting heat-killed CP to the CP-specific lines CG-21, K-43, and K-66 (Figure 11 and Table 13).

It has been reported that some T cell clones preferentially collaborate with a particular APC type (Katz and Feldmann, 1983). Although the T cell lines used here were polyclonal, the apparent greater efficiency of MØ for CP presentation could have reflected selective expansion of MØ-prefering T cell clones, rather than an inherent deficiency of DC in presenting whole bacteria. Since T cell lines established from CFA-immunized mice proliferated to both PPD and BCG, this possibility could be assessed using a single line displaying specificity for both a soluble and particulate antigen. The results essentially confirmed the observations made with the individual T cell lines (Table 14). At lower cell concentrations, MØ and DC were

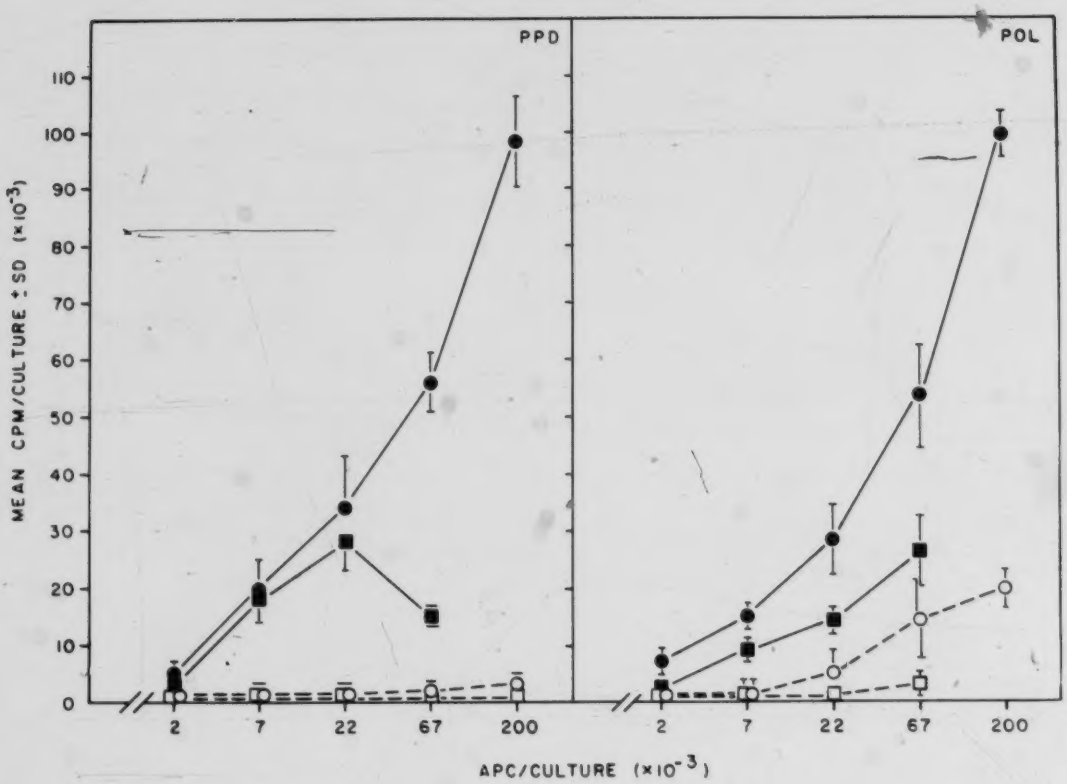

Figure 10. Comparison of splenic MØ and DC for PPD and POL presentation. The indicated numbers of CBA/CaJ splenic MØ (squares) or DC (circles) were cultured with a CBA/CaJ T cell line specific for PPD (left panel) or POL (right panel). APC were pulsed 100 μg/ml PPD (left panel, closed symbols) or POL (right panel, closed symbols) for 2 hr at 37° C, or used unpulsed (open symbols). Background responses of PPD-specific and KLH-specific T cell lines without APC were 0.3 and 0.2 cpm (x 10^{-3})/culture, respectively.

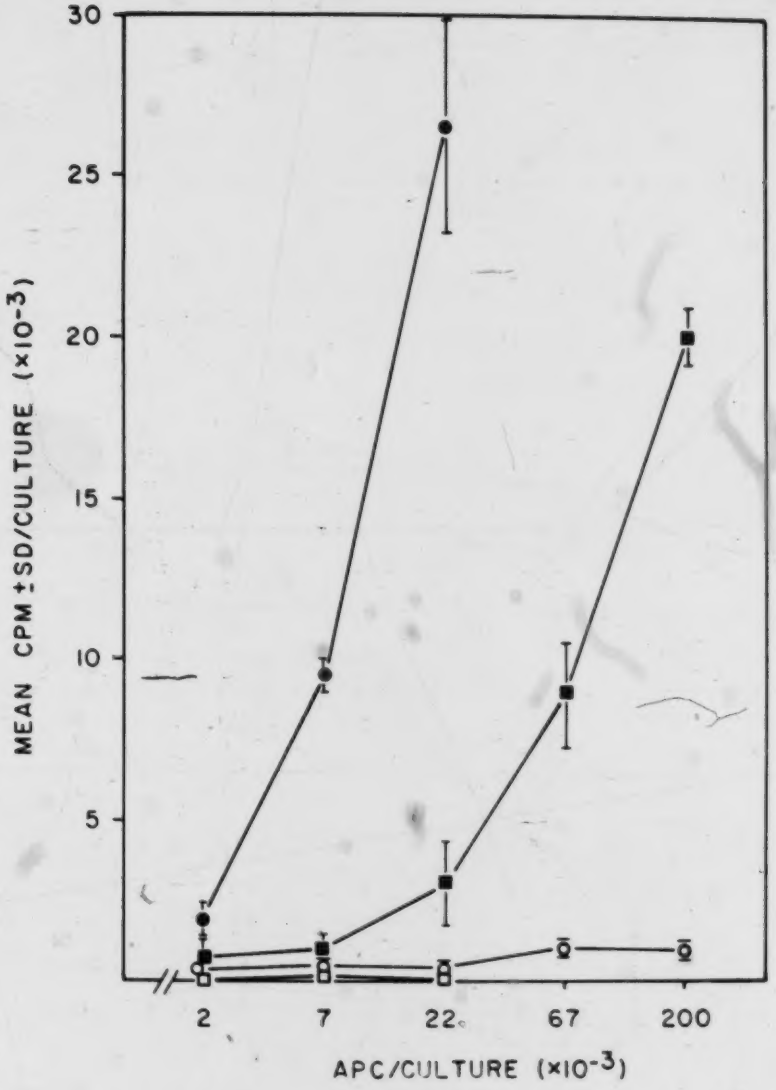

Figure 11. Comparison of splenic MØ and DC for CP presentation. The indicated numbers of CBA/CaJ splenic MØ (circles) or DC (squares) were cultured with a CBA/CaJ CP-specific T cell line. APC were either pulsed with CP (100 μg/ml for 2 hr at 37° C, closed symbols) or unpulsed (open symbols). Background response without APC was 0.3 cpm (x 10^{-3})/culture.

TABLE 13

Presentation of CP to CP-Specific T Cell Lines by Splenic MØ and DC

Expt./CP Line[a]	APC/Culture ($\times 10^{-3}$)	Proliferative Response[b] (mean Δcpm ± SD/culture ($\times 10^{-3}$))	
		DC	MØ
Expt. 1/CG-21	0.9	< 1	1
	2.7	1	5 ± 1
	8	8 ± 1	21 ± 2
	23	19 ± 2	28 ± 1
Expt. 2/K-43	10	3 ± 1	5
	33	9 ± 2	18 ± 3
	100	19 ± 2	44 ± 2
	300	31 ± 2	ND[c]
Expt. 3/K-66	2.7	5	10 ± 1
	8	14 ± 2	22 ± 2
	23	26 ± 2	46 ± 1
	70	42 ± 4	ND
	210	49 ± 1	ND

[a] Background proliferation for lines CG-21, K-43 and K-66 was 0.2, 0.1, and 0.2 cpm ($\times 10^{-3}$)/culture, respectively.

[b] Responses to unpulsed APC have been subtracted. SD < 1000 cpm are not shown.

[c] ND, not determined.

TABLE 14

Comparison of PPD and BCG Presentation by Splenic MØ and DC

APC[a]		Proliferative Response[b] (mean Δcpm ± SD/culture (x 10^{-3}))	
Type	No./Culture (x 10^{-3})	PPD	BCG
	0	0.1	0.2
DC	0.7	2 ± 1	< 0.1
	2.2	8 ± 2	0.2
	6.7	16 ± 2	3 ± 1
	22	32 ± 4	16 ± 3
	67	53 ± 5	32 ± 5
MØ	0.7	1	0.2
	2.2	5 ± 1	2 ± 1
	6.7	13 ± 2	10 ± 1
	22	30 ± 1	31 ± 1
	67	32 ± 1	38 ± 4

[a] MØ and DC were purified from CBA/CaJ spleen cells.

[b] The PPD-specific or BCG-specific proliferative responses induced by PPD- or BCG-pulsed MØ or DC in a CBA/CaJ T cell line were assessed. The T cell line was derived from mice immunized with CFA, and was reactive to both PPD and BCG. The responses to APC without antigen have been subtracted. SD < 1000 cpm are not shown.

comparable for PPD presentation, whereas at high cell concentrations, DC were more effective. In contrast, MØ were more effective than DC at presenting whole washed BCG organisms, especially at low cell concentrations. These results provide further support for the notion that DC are relatively inefficient as APC for whole bacterial organisms.

Antigen Presentation by Activated BM-MØ

The observation that splenic MØ were more efficient than DC at presenting whole bacteria suggests that the phagocytic and degradative activities of MØ are more suited to the processing of particulate antigens. The validity of this notion was further evaluated using activated BM-MØ subpopulations with the prediction that the best antigen-presenting BM-MØ (i.e., the smallest) should be more effective than splenic MØ at presenting particulate antigens, by virtue of the maturity and greater phagocytic activity of the former cells (Adams and Hamilton, 1984). This was indeed the case (Figure 12). About one-third as many of the smallest BM-MØ (fraction A) as splenic MØ were sufficient to induce the same level of CP-specific T cell proliferation. Consistent with earlier results (Figure 11 and Table 14), DC were about three times less efficient (on a per cell basis) than splenic MØ as APC in this system (Figure 12). When BM-MØ were compared with splenic MØ and DC for the presentation of KLH, a different pattern emerged (Figure 13). In contrast to CP presentation, DC were slightly better than the smallest activated BM-MØ for the presentation of this soluble protein antigen (Figure 13). As observed with MLR activation (Figures 7 and 8) and CP presentation (Figure 12), larger BM-MØ had lower activities.

Antigen Presentation by the DC-Like Cell Line P388AD.4

In view of the lack of phagocytic activity by DC, it is surprising that they could present whole bacteria at all. MØ contaminants in the DC fraction could have generated enough processed antigen to be presented by DC, even though such contamination was minimal immediately after DC purification (< 1% Fc rosettes), and remained so after 3 days in culture (< 0.5% of the rosettes in the MØ fraction). Thus, there was no evidence for development of

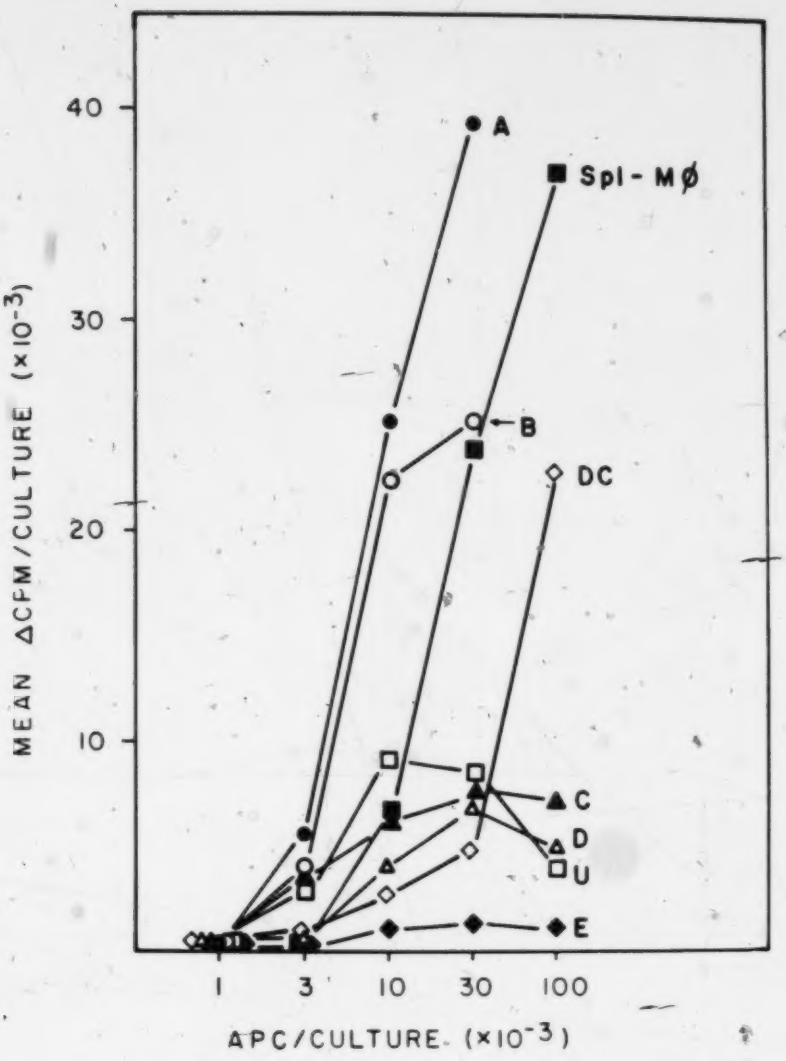

Figure 12. Comparison of splenic MØ, DC and activated BM-MØ for presentation of CP. The proliferative response of a CBA/CaJ CP-specific T cell line to CP (12 μg/ml) presented by CBA/CaJ splenic MØ, DC, and activated, size-fractionated BM-MØ (A-E) was assessed. Unfractionated (U) BM-MØ were also tested. Standard deviations were < 10% of the mean, and background responses to APC alone have been subtracted. Sedimentation velocities of BM-MØ fractions in mm/hr were: A, 3.0-6.2; B, 6.2-7.7; C, 7.7-9.8; D, 9.8-11.5; and E, 11.5-14.4. The percentages of cells recovered in fractions A to E were: 14.5, 17.1, 28.9, 18.4, and 21.1.

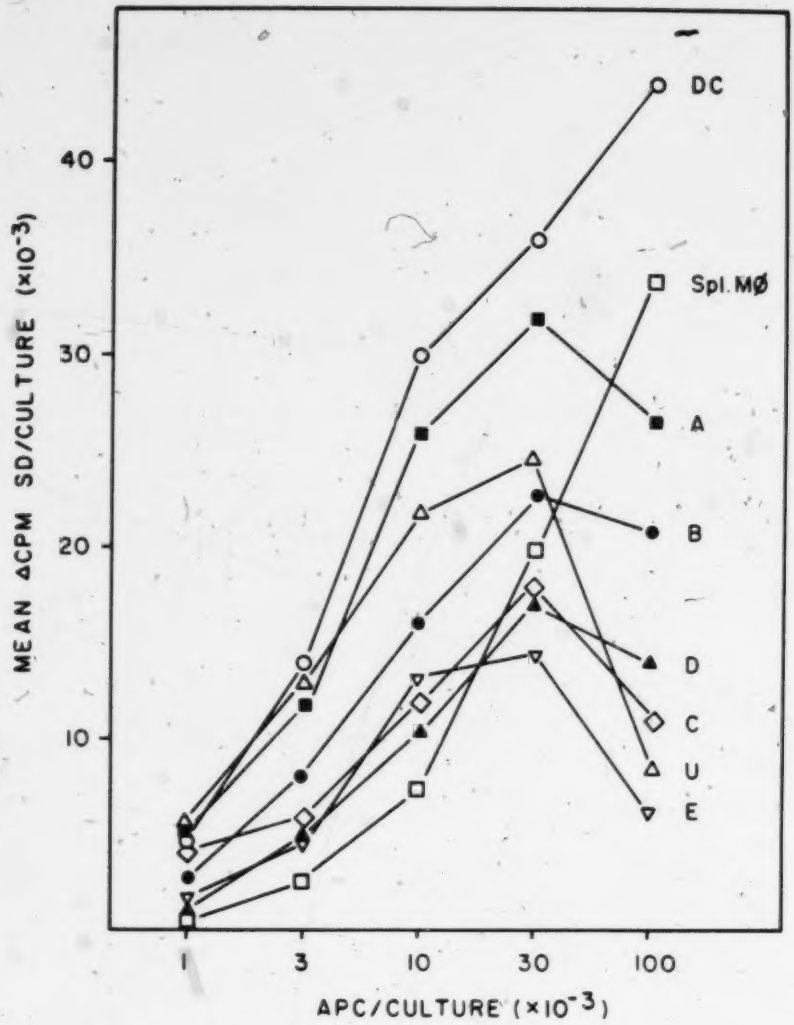

Figure 13. Comparison of splenic MØ, DC, and activated BM-MØ for presentation of KLH. The proliferative response of a CBA/CaJ KLH-specific T cell line to KLH (50 μg/ml) presented by CBA/CaJ splenic MØ, DC, or activated, size-fractionated BM-MØ was assessed. Unfractionated (U) BM-MØ were also tested. Standard deviations were < 10% of the mean, and background responses to APC alone have been subtracted. Sedimentation velocities of BM-MØ fractions in mm/hr were: A, 3.5-6.2; B, 6.2-8.0; C, 8.0-9.9; D, 9.9-11.9; and E, 11.9-14.4. The percentages of cells recovered in fractions A to E were: 18.9, 22.8, 21.5, 19.9, and 17.1.

FcR^+ MØ from FcR^- precursors in the non-rosetting DC fraction. Nonetheless, the possible role of MØ contaminants could not be rigorously excluded. Therefore, P388AD.4, a nonphagocytic, FcR^-, Ia^+, DC-like cell line derived from the DBA/2 P388 leukemia (Cohen and Kaplan, 1981), was used to determine whether a pure population of nonphagocytic cells could process and present whole bacteria. P388AD.4 could indeed present CP and BCG, albeit much less efficiently than unfractionated splenic APC (Table 15). Consistent with the results of Cohen and Kaplan (1981,1983), P388AD.4 stimulated syngeneic and allogeneic MLR (Table 16) and presented soluble antigens to T cell lines (Table 17). Thus, poor CP presentation by P388AD.4 did not reflect generalized APC dysfunction. These results confirm that nonphagocytic APC are relatively inefficient for the processing and presentation of whole bacterial organisms, especially in the absence of the potential for contamination by MØ (Table 15).

Flow Cytometric Analysis of Ia Expression

Previous work has indicated that the product of [Ia] x [antigen] is a critical factor in T cell activation (Matis *et al*, 1983; Lechler *et al*, 1985). To obtain precise quantitative data on Ia levels, splenic MØ, DC, and BM-MØ subsets were analyzed by indirect immunofluorescence and flow cytometry to determine whether the observed functional differences among APC subsets correlated with differences in Ia expression.

Monoclonal anti-I-A^k and anti-I-A^d (specificity control) antibodies were used as serum-free culture supernatants of the 10-2.16 and MKD6 B cell hydridomas, respectively. The specificity of these antibodies was verified in preliminary ELISA assays done on MØ monolayers (Table 4). The results in Figure 14 show that the binding of anti-I-A^k to unactivated H-2^k BM-MØ was very much lower than to activated H-2^k BM-MØ, whereas activation had little effect on the expression of the class I MHC antigen H-$2K^k$. The profiles of negative control cells (treated with FITC-SAMIG only) were almost identical to the specificity controls. Thus, nonspecific binding (to FcR) was negligible. The channel above which no more than 5% of negative (and specificity) control cells fell was selected as the

TABLE 15

Presentation of Particulate Antigens by the DC-Like Cell Line P388AD.4

APC		Proliferative Response[a] (mean Δcpm ± SD/culture (x 10^{-3}))	
Type	No./Culture (x 10^{-3})	CP	BCG
	0	0.1	0.2
Spleen Cells[b]	100	12.7 ± 1.5	5.6 ± 1.2
	300	28.8 ± 0.5	45.6 ± 7.7
	1000	23.7 ± 4.4	94.5 ± 4.8
P388AD.4[c]	3	ND[d]	0.1 ± 0.1
	10	0.1 ± 0.1	0.7 ± 0.4
	30	1.3 ± 0.6	2.0 ± 0.1
	100	3.2 ± 0.5	5.3 ± 0.4

[a] DBA/2J T cell lines specific for CP or BCG were cultured with CP or BCG at 10 μg/ml and 20 μg/ml respectively. The responses to APC without antigen have been subtracted. SD < 100 cpm are not shown.

[b] DBA/2J, irradiated (2000 rad).

[c] Mitomycin C treated.

[d] ND, not determined.

TABLE 16

Syngeneic and Allogeneic MLR Stimulation by P388AD.4

APC		Proliferative Response[a] (mean Δcpm ± SD/culture (x 10^{-3}))	
Type	No./Culture (x 10^{-3})	DBA/2J[b]	CBA/CaJ[b]
	0	0.8	1.2
Spleen Cells[c]	67	1.2 ± 0.2	11.0 ± 1.5
	220	2.4 ± 0.2	45.7 ± 5.4
	670	3.6 ± 0.5	82.5 ± 4.2
	2000	4.2 ± 0.6	31.7 ± 2.7
P388AD.4[d]	3	2.6 ± 0.4	27.2 ± 5.6
	10	6.4 ± 0.9	54.6 ± 6.5
	30	13.3 ± 1.6	72.3 ± 17.2
	100	25.1 ± 4.3	95.9 ± 14.6

[a] SD < 100 cpm are not shown.

[b] Syngeneic DBA/2J or allogeneic CBA/CaJ adherence-depleted spleen cell responders (5 x 10^5/culture).

[c] DBA/2J, irradiated (2000 rad).

[d] Mitomycin C treated.

TABLE 17

Presentation of Soluble Antigens by P388AD.4

APC		Proliferative Response[a] (mean Δcpm ± SD/culture (x 10^{-3}))	
Type	No./Culture (x 10^{-3})	KLH[b]	PPD[b]
	0	0.3	0.5
Spleen Cells[c]	30	1.4 ± 0.4	32.9 ± 8.6
	100	6.1 ± 0.9	46.3 ± 3.7
	300	29.3 ± 2.2	67.4 ± 6.3
	1000	46.9 ± 1.3	69.4 ± 3.6
P388AD.4[d]	3	1.1 ± 0.1	44.3 ± 13.6
	10	3.5 ± 0.3	54.1 ± 1.8
	30	35.0 ± 5.8	75.0 ± 6.4
	100	ND[e]	93.9 ± 1.3

[a] Responses to APC without antigen have been subtracted. SD < 100 cpm are not shown.

[b] DBA/2J KLH-specific or PPD-specific T cell lines were cultured with the indicated APC and KLH or PPD, respectively, at 25 μg/ml.

[c] DBA/2J, irradiated (2000 rad).

[d] Mitomycin C treated.

[e] ND, not determined.

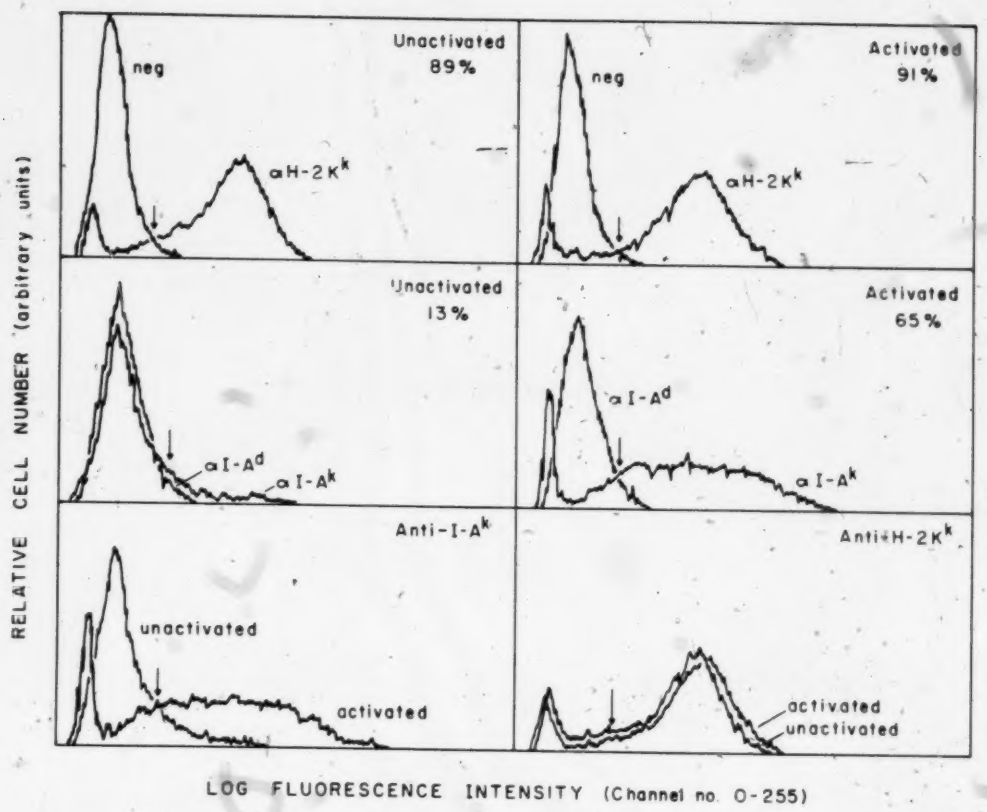

Figure 14. Flow cytometric analysis of Ia expression on unactivated and activated BM-MØ. Unfractionated CBA/CaJ BM-MØ were analyzed for expression of H-2K^k (16-3-1N) and I-A^k (10-2.16) by indirect immunofluorescence. Cells were also treated with anti-I-A^d (MKD6) as a specificity control. The channel above which no more than 5% of the negative control cells fell was selected as the minimum channel defining positive cells and is indicated with an arrow in each panel. Numbers in the top right hand corners of the first four panels refer to the percentage of positive cells in the population.

minimum channel defining I-A^k positive cells, and is indicated with an arrow in each panel. Using this criterion, unactivated BM-MØ had few (13%) Ia^+ cells, in accordance with their inactivity in MLR stimulation. Both the percentage and the mean fluorescence intensity (Ia density) of Ia^+ BM-MØ were dramatically increased by activation.

The results of flow cytometric analysis of splenic MØ, DC and activated BM-MØ fractions treated with anti-I-A^k or anti-I-A^d antibodies are shown in Figure 15. Negative controls treated with the fluorescent second antibody only were indistinguishable from anti-I-A^d treated cells and are not shown. The mean fluorescence intensities of the anti-I-A^k-treated cells exhibiting fluorescence above the threshold were 3-7 times greater than those of cells treated with anti-I-A^d or no antibody (legend, Figure 15). A better estimate of the I-A^k-specific fluorescence for each cell type is the relative fluorescence index, which represents the difference in degree of brightness of a single cell population treated with different antibodies. Calculation of this figure (Weeks *et al*, 1984) shows that the anti-I-A^k-treated cells ranged from 3 (fraction B) to 18 (DC) times brighter than the same cells treated with anti-I-A^d. In view of the autofluorescence of MØ, this represents a substantial level of I-A^k-specific fluorescence.

The most effective APC (i.e., DC, splenic MØ and small BM-MØ) had the highest level of surface Ia as represented by the mean fluorescence intensity (Figure 15). However, large BM-MØ expressed a substantial amount of Ia, but minimal APC functions (Figures 7,8,12, and 13), possibly due to suppressive activity (Lee and Wong, 1982). Small MØ (BM or splenic) were not immunosuppressive (Lee and Wong, 1982), had Ia levels similar to DC, yet were poor stimulators of syngeneic MLR (Figures 6 and 8). Conversely, DC, which had high levels of surface Ia, were poor APC for presentation of particulate antigens (Tables 13 and 14, Figures 11 and 12). Thus, there was no simple correlation between surface Ia expression and the T cell stimulatory capacity of various APC.

All cell types contained a population of Ia^- cells which are presumably inactive as APC (Figure 15). Since the splenic and small BM-MØ contained > 98% and 100% MØ respectively (by morphologic, phagocytic, and cytochemical criteria), the Ia^- cells must be MØ and not

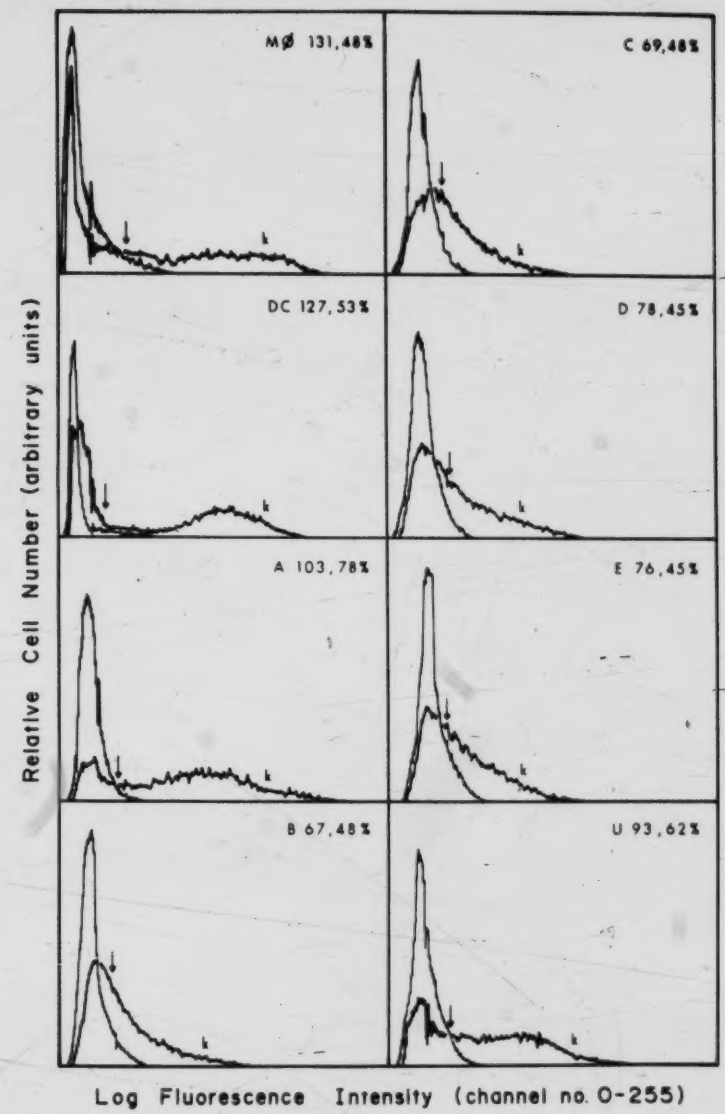

Figure 15. Flow cytometric analysis of Ia expression on APC subsets. CBA/CaJ splenic DC and MØ, and activated, size-fractionated (A-E) or unfractionated (U) BM-MØ were analyzed by indirect immunofluorescence for expression of I-A^k (thick lines, labeled k) or I-A^d (thin lines, unlabeled) as a specificity control. The profiles of negative control cells (treated with FITC-SAMIG only) were identical to those of the specificity controls and are not shown. The channel above which no more than 5% of the negative control cells fell was selected as the minimum channel defining positive cells and is indicated by an arrow in each panel. Numbers in the top right corner of each panel refer to the mean fluorescence intensity and percent positive cells respectively. The mean fluorescence intensities of specificity controls were: splenic MØ, 30; DC, 18; A, 22; B, 23; C, 27; D, 29; E, 28; and U, 25. Sedimentation velocities and percentages of cells recovered in fractions A-E are given in the legend for Figure 5.

other cell types. In contrast, the DC preparation routinely contained 20-30% of other cell types (granulocytes, lymphocytes, and stromal cells, but not MØ) identified morphologically (Table 3). To determine whether only one or both populations in the DC fraction contained Ia$^+$ cells, two parameter histograms of log 90° (L90) light scatter *vs* log fluorescence were collected. The former technique distinguishes lymphocytes from DC or MØ based on differences in cell-surface characteristics (e.g., membrane ruffling), nucleus to cytoplasm ratio, granularity, and size (Salzman *et al*, 1975; Thompson *et al*, 1985). The results show that the population displaying the greatest amount of L90 light scatter (i.e., DC) contained all the Ia$^+$ cells (Figure 16). This is consistent with previous demonstrations that all DC are Ia$^+$ (Steinman *et al*, 1979). In contrast, MØ are heterogeneous with respect to Ia expression (reviewed in Unanue, 1984). Accordingly, splenic MØ and BM-MØ fractions showed a continuum of Ia expression ranging from negative to maximum. Furthermore, Ia$^+$ and Ia$^-$ cells in the MØ fractions were not distinguishable by L90 light scatter profiles (Figure 16), confirming that the MØ populations used in these studies were pure. The level of impurity of the DC fraction (20-30% in most experiments) should not be critical, since the contaminating cell types were Ia$^-$, and $<$ 1% MØ were present. Moreover, the APC functions were evaluated over a wide range of cell concentrations (Figures 6-8, 10-13, Tables 13 and 14), and the observed functional differences were too great to be accounted for by the impurities.

Antigen Uptake Studies

To test the possibility that the observed variation among APC subsets in antigen-presentation was due to differences in their ability to bind antigen, the uptake of ^{125}I-labeled soluble and particulate antigens was measured. Large BM-MØ were found to take up more CP than small MØ (fraction A, Table 18), possibly because of the greater phagocytic activity of the former cells. No such difference was observed for KLH (Table 18), which could have been taken up mainly by pinocytosis. Splenic MØ and DC took up similar amounts of CP (Table 19). In contrast, P388AD.4 took up about 10 times less CP during a 2 hr antigen pulse than did BM-MØ and the MØ-like lines P338D$_1$ and J774A-1 (0.1-0.3% *vs* 2-3% of the amount

Figure 16. Flow cytometric analysis of splenic DC, MØ, and activated BM-MØ for L90 light scatter and Ia expression. The same cells used for the experiment shown in Figure 15 were analyzed simultaneously for L90 light scatter and fluorescence, and the results are displayed as 2 parameter histograms. For each cell type, the left, middle, and right panels show the profiles of the negative (treated with FITC-SAMIG only), specificity (treated with anti-I-A^d), and anti-I-A^k-treated cells, respectively. The sedimentation velocities and percentages of cells recovered in fractions A-E are given in the legend for Figure 5. This figure is continued on the next page.

Figure 16. (Continued)

TABLE 18

Uptake of ^{125}I-Labeled Antigens by BM-MØ and MØ-Like and DC-Like Tumors

Cells[a]	Antigen Uptake[b] (mean cpm ± SD/culture (x 10^{-3}))	
	^{125}I-CP	^{125}I-KLH
A	9.6 ± 2.3	11.9 ± 1.6
B	20.5 ± 2.4	10.2 ± 2.7
C	27.0 ± 1.9	10.3 ± 3.0
D	22.2 ± 1.8	13.0 ± 1.2
U	23.2 ± 1.3	13.4 ± 2.0
P388AD.4	2.5	9.8 ± 3.1
$P388D_1$	14.7 ± 1.7	15.2 ± 1.9
J774A-1	30.7 ± 1.8	19.8 ± 3.2
none	1.3	0.6

[a] BM-MØ were grown for 6 days and fractionated by velocity sedimentation (A-D) or left unfractionated (U), and activated overnight in DMEM-10% FBS containing 3 μg/ml LPS and 10% Ia-inducing lymophokines. Sedimentation velocities in mm/hr were: A, 2.2-6.5; B, 6.5-7.8, C, 7.8-10.0; D, 10.0-12.3. The percentages of cells recovered in fractions A to D were: 16.4, 22.1, 34.6, and 26.9. The DC-like tumor, P388AD.4 and the MØ-like tumors, $P338D_1$ and J774A-1, were also used. SD < 1000 cpm are not shown.

[b] Cells (10^5 in 0.2 ml/culture) adhering to flat-bottomed tissue culture wells (96-well trays) were incubated with 14 μg ^{125}I-CP (29,000 cpm/μg) or 10 μg ^{125}I-KLH (60,000 cpm/μg) for 2 hr. After 4 washes with warm medium, cells were solubilized with 0.1 ml of 0.25% SDS and aliquots were taken for gamma counting.

TABLE 19

Uptake of ^{125}I-Labeled CP Organisms by Splenic MØ and DC

Cells	Chloroquine	Antigen Uptake[a] (mean cpm ± SD/culture (x 10^{-3}))
DC	-	365 ± 16
	+	374 ± 25
MØ	-	441 ± 27
	+	400 ± 12

[a] Triplicate cultures (7 x 10^5 cells/0.5 ml/culture) were incubated with 70 μg ^{125}I-labeled CP/ml (2.2 x 10^5 cpm/μg) ± 0.3 mM chloroquine for 2 hr. The cells were washed three times and transferred to new tubes for the determination of radioactivity.

added, Table 19). Once again, no such difference was observed for the uptake of KLH. P388AD.4 cells were also less active in the degradation of CP than were BM-MØ (Table 20). P388AD.4 released more antigen during the post-pulse culture phase than did BM-MØ, but no acid-soluble material was detected. By contrast, BM-MØ released more acid-soluble than acid-insoluble material during the same culture period, in accordance with their known degradative capacity.

These studies suggest that the amount of antigen taken up by various APC populations is not a limiting factor with respect to APC function in this system. Although P388AD.4 exhibited a selective deficiency in CP uptake during a 2 hr pulse, it was not an effective APC for CP even when this antigen was present throughout the culture period (Table 15). Thus, although poor antigen uptake may be a contributory factor, the decreased ability of P388AD.4 to catabolize CP as compared to BM-MØ may be more relevant to its inefficiency as an APC for CP.

E. Mechanism of Antigen-Presentation by DC and MØ

Chloroquine, a lysosomotropic agent, can block the processing and presentation of soluble protein (Chestnut *et al*, 1982a; Lee *et al*, 1982) and particulate (Ziegler and Unanue, 1982) antigens, but not small peptides (such as PPD) or integral membrane Ia (for MLR stimulation) (Lee *et al*, 1982; Scala and Oppenheim, 1983; Finnegan *et al*, 1985); both are apparently presented without modification. Because the splenic APC used were unfractionated, it was impossible to tell whether the cell types themselves (DC *vs* MØ) or individual functions of each cell type were distinguishable on the basis of chloroquine sensitivity. Accordingly, DC and MØ fractions were characterized with respect to antigen-presenting function and chloroquine sensitivity. The results in Table 21 show the difficulties encountered if primed LN T cells were used as responders. As expected from previous work (Lee *et al*, 1982), MØ were capable of inducing antigen-dependent proliferation of primed LN T cells above background levels, and preincubation with 0.3 mM chloroquine for 2 hr (followed by washing) prior to culture suppressed the presentation of POL (MW millions) to a greater extent than that of

TABLE 20

Uptake and Degradation of ^{125}I-CP by BM-MØ and P388AD.4

Cells[a]	Antigen Uptake[b] (cpm ± SD /Culture (x 10^{-3}))	% Antigen (after 1.5 hr) in[c]		
		Cells	Supernatant	
			TCA-Soluble	TCA-insoluble
A	44.2 ± 1	69 ± 2	12	3
B	53.9 ± 2	76 ± 1	12	2
C	53.0 ± 2	76 ± 7	12	3
D	52.5 ± 1	76 ± 5	11	3
E	56.1 ± 2	74 ± 5	11	2
U	54.1 ± 3	80 ± 2	12	2
P388AD.4	2.4	63 ± 1	< 1	29

[a] BM-MØ were grown for 6 days and fractionated by velocity sedimentation (A-E) or left unfractionated (U), irradiated and activated overnight as usual. Sedimentation velocities in mm/hr were: A, 3.5-6.2; B, 6.2-8.0; C, 8.0-9.9; D, 9.9-11.9; E, 11.9-12.3. The percentages of cells recovered in fractions A to E were: 12.7, 23.5, 28.6, 22.4, and 12.8. P388AD.4 cells (mitomycin C treated) were also used.

[b] Two groups of cells (10^5 cells in 0.2 ml/well, 4 wells/group) adhering to flat-bottomed tissue culture wells (96-well trays) were pulsed with 14 μg ^{125}I-labeled CP (10^5 cpm/μg) for 2 hr. After 4 washes with warm medium, one group of cells was solubilized with 0.1 ml of 0.25% SDS for determination of antigen uptake. SD < 1000 cpm not shown.

[c] The second group of cells was incubated for a further 1.5 hr, and the amounts of antigen remaining in cells and supernatant fluids (soluble or insoluble in 10% trichloroacetic acid, TCA) were measured and expressed as a percentage of the amount initially cell-bound (column 2). SD < 1% not shown.

TABLE 21

Presentation of Soluble Antigens to LN T Cells - Effect of Chloroquine

APC[a]			Proliferative Response[b] (mean cpm ± SD/culture (x 10^{-3}))		
Type	Chloroquine Treatment	No./Culture (x 10^{-3})	Unpulsed	PPD	POL
		0	0.3		
DC	-	22	14 ± 2	24 ± 8	36 ± 5
		67	30 ± 6	32 ± 4	52 ± 8
		200	18 ± 6	41 ± 4	48 ± 5
	+	22	4 ± 1	19 ± 5	20 ± 3
		67	25 ± 1	23 ± 1	35 ± 3
		200	27 ± 7	33 ± 6	39 ± 6
MØ	-	22	1	23 ± 5	3 ± 1
		67	7 ± 3	62 ± 13	29 ± 3
		200	7 ± 1	60 ± 1	80 ± 11
	+	22	1	2 ± 1	2 ± 1
		67	1	9 ± 2	2 ± 1
		200	2	44 ± 5	3 ± 2

[a] MØ and DC were purified from CBA/CaJ spleen cells, and treated with 0.3 mM chloroquine at 37° C, or left untreated. APC were either unpulsed, or pulsed with PPD or POL.

[b] Responders were nylon wool-purified LN T cells from mice that had been immunized in the hind footpads with POL/CFA 8 days previously. The maximal responses of unfractionated LN cells to PPD and POL were 64 ± 9 and 39 ± 4 cpm (x 10^{-3}) respectively.

PPD (MW 2000 to 6000). No loss in cell viability was observed after chloroquine treatment. However, DC routinely induced high background responses that were virtually unaffected by chloroquine (Table 21), and could be identical to the syngeneic MLR obtained with spleen responder cells (Figures 6 and 8). Therefore, the response stimulated by POL-pulsed, chloroquine-treated DC remained high.

When T cell lines were used as responders, the problem with high backgrounds was eliminated, but the effect of chloroquine on antigen presentation was very similar to that obtained with primed LN T cells. Thus the presentation of OVA (Table 22) or CP organisms (Table 23) by DC and MØ to T cell lines was abrogated completely by chloroquine pretreatment, whereas the induction of syngeneic and allogeneic MLR was affected to a lesser extent (Table 23).

The inhibition of APC functions was dependent on the concentration of chloroquine included during the antigen-pulse (Table 24). Whereas 0.1 mM chloroquine had only marginal effects on the presentation of BCG by DC and MØ, 0.3 mM chloroquine had a much more pronounced effect (Table 24). The optimal chloroquine concentration varied slightly in different experiments, but was usually between 0.3 mM and 0.5 mM. On the occasions when the presentation of xenogeneic antigens was only partially inhibited, MLR activation was unaffected (data not shown). Thus, selective inhibition of antigen-presentation was obtainable under stringent conditions.

The paucity of lysosomes in DC suggests that the presenting function of such cells may be less chloroquine sensitive than that of MØ. A T cell line reactive to PPD and BCG was used to investigate this question. Normal and chloroquine-treated (0.3 mM) MØ and DC were compared as APC for PPD and BCG presentation. The effect of chloroquine pretreatment on DC varied according to the antigen being presented (Figure 17). Consistent with previous results (Lee *et al*, 1982; Table 21), chloroquine pretreatment of DC only moderately inhibited PPD presentation (Figure 17). In contrast, the presentation of BCG by both DC and MØ was almost completely inhibited by chloroquine pretreatment. If MØ and DC were pretreated with 0.1 mM chloroquine, PPD presentation by DC was virtually unaffected, whereas BCG

TABLE 22

Effect of Chloroquine on Presentation of OVA

APC[a]			Proliferative Response[b] (mean cpm ± SD/culture (x 10^{-3}))	
Type	Chloroquine Treatment	No./Culture (x 10^{-3})	Unpulsed	OVA-pulsed
MØ	-	11	0.2	1
		33	0.3	3
		100	1	12 ± 2
		300	ND[c]	ND
MØ	+	11	0.1	0.4
		33	0.2	0.2
		100	0.5	0.2
		300	ND	ND
DC	-	11	0.5	1
		33	0.9	5 ± 1
		100	2	15 ± 2
		300	3	19 ± 3
DC	+	11	0.3	0.2
		33	0.5	0.1
		100	2	0.4
		300	3	1

[a] MØ and DC were purified from CBA/CaJ spleen cells.

[b] Proliferative response of a CBA/CaJ OVA-specific T cell line was assessed. Background proliferation without APC was 0.2 cpm (x 10^{-3})/culture. SD < 1000 cpm are not shown.

[c] ND, not determined.

TABLE 23

Selective Inhibition of Xenogeneic Antigen Presentation by Chloroquine

APC[a]		Proliferative Response (mean cpm ± SD/culture (x 10^{-3}))			
		MLR[b]		CP presentation[c]	
Type	No./Culture (x 10^{-3})	Syngeneic	Allogeneic	Unpulsed	CP-pulsed
	0	2 ± 1	8 ± 1	0.2	
DC	2	6 ± 1	82 ± 20	0.4	1 ± 1
	7	13 ± 2	145 ± 9	0.5	1 ± 1
	22	34 ± 7	109 ± 6	0.3	4
	67	89 ± 7	82 ± 9	1	10 ± 1
	200	121 ± 11	61 ± 14	1	20 ± 1
DC	2	4	30 ± 4	0.1	1
Chloroquine-	7	9 ± 1	52 ± 10	0.3	1
treated	22	13 ± 2	90 ± 6	0.2	0.4
	67	32 ± 3	167 ± 2	0.3	1 ± 1
	200	86 ± 9	140 ± 18	0.5	0.5
MØ	0.7	3 ± 1	31 ± 8	0.3	0.2
	2	2	70 ± 4	0.2	2
	7	7 ± 1	133 ± 19	0.4	10 ± 1
	22	14 ± 2	104 ± 14	0.6	26 ± 3
MØ	0.7	2	7 ± 2	0.2	0.4
Chloroquine-	2	2 ± 1	13 ± 4	0.2	0.4
treated	7	4 ± 1	37 ± 4	0.4	0.2
	22	8 ± 2	96 ± 3	1	0.3

[a] MØ and DC were purified from CBA/CaJ spleen cells.

[b] Syngeneic CBA/CaJ or allogeneic BALB/cCR spleen cell responders (4 x 10^5/culture).

[c] CBA/CaJ CP-specific T cell line; SD < 1000 cpm are not shown.

TABLE 24

Inhibition of Antigen Presentation by Chloroquine is Dose-Dependent

APC[a]		Proliferative Response[b] (mean Δcpm ± SD/culture (x 10^{-3}))		
Type	No./Culture (x 10^{-3})	untreated[c]	0.1 mM[c]	0.3 mM[c]
MØ	0.7	0.2	< 0.1	< 0.1
	2.2	2 ± 1	0.4	< 0.1
	6.7	10 ± 1	3	0.3
	22	31 ± 1	15 ± 1	3 ± 1
	67	38 ± 4	28 ± 4	9 ± 2
DC	0.7	< 0.1	< 0.1	< 0.1
	2.2	0.2	< 0.1	< 0.1
	6.7	3 ± 1	3 ± 1	0.5
	22	16 ± 3	10 ± 2	2
	67	32 ± 5	22 ± 2	7 ± 2

[a] MØ and DC were purified from CBA/CaJ spleen cells.

[b] The proliferative responses induced by BCG-pulsed APC in a CBA/CaJ BCG-specific T cell line were assessed. The responses to APC without antigen have been subtracted. SD < 100 cpm are not shown.

[c] APC were either untreated, or treated with the indicated concentration of chloroquine during the antigen pulse.

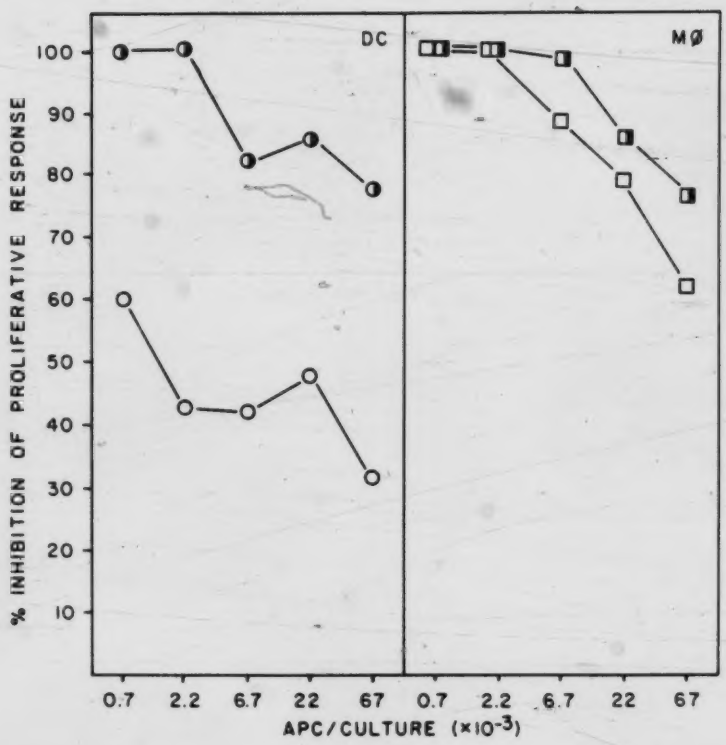

Figure 17. Effect of chloroquine on the presentation of PPD and BCG by splenic DC and MØ. CBA/CaJ splenic DC (left panel) or MØ (right panel) were cultured with a CBA/CaJ T cell line specific for both PPD and BCG. The antigen-specific proliferative response was determined as the difference between the response to unpulsed and PPD-pulsed (open symbols) or BCG-pulsed (half-closed symbols) DC or MØ. Results represent the proliferative response induced by chloroquine-treated (0.3 mM) DC and MØ expressed as a percentage of the response induced by untreated DC and MØ, respectively.

presentation by either MØ or DC was moderately inhibited (data not shown). Thus, a differential sensitivity of MØ and DC to the effects of chloroquine could be discerned for the presentation of some antigens, but not others. In general, the sensitivity to chloroquine increased with the size of the antigen being presented for both MØ and DC.

Chloroquine had no effect on antigen uptake (Tables 19 and 25) in agreement with previous studies (Grey *et al*, 1982; Lee *et al*, 1982; Ziegler and Unanue, 1982). In contrast to results obtained with BM-MØ (Lee *et al*, 1982), the low capacities of splenic MØ and DC to degrade antigens into acid-soluble material was unaffected by chloroquine (Table 25). Most of the antigen remained cell-bound after 1.5 hr. Of the released material, the majority was acid-precipitable, but it was impossible to tell whether any degradation had taken place. These results suggest that the processed antigen relevant to immune stimulation represents a very small fraction of the total antigen initially taken up by APC.

F. Mechanism of Particulate Antigen Presentation by DC and DC-like lines

Although MØ were much more effective than DC or P388AD.4 at presenting whole bacterial organisms, the ability of nonphagocytic APC to perform this function at all was unexpected. Much of this low level of activity could have resulted indirectly from the pinocytosis of soluble antigen shed from the bacteria. Consistent with this, immunogenic material was present in the filtrates of stored BCG and CP suspensions (Tables 26 and 27); the CP filtrate was presented much more efficiently by P388AD.4 than whole CP organisms at optimal concentrations (Table 27).

To directly assess the role of shed bacterial antigens in CP-specific T cell activation by different APC subsets, CP organisms were fixed with glutaraldehyde to reduce or eliminate antigen shedding (Golding and Singer, 1984) with the prediction that this should selectively reduce the immunogenicity of CP presented by nonphagocytic APC when compared to presentation by phagocytic APC. In agreement with this, DC and P388AD.4 were found to be much less efficient than MØ in presenting glutaraldehyde-fixed CP (Figure 18). This was particularly striking with P388AD.4, possibly because there were no MØ contaminants to

TABLE 25

Effect of Chloroquine on Uptake and Degradation of ^{125}I-Labeled Antigens by Splenic MØ and DC

Cells	Antigen	Chloroquine	Antigen Uptake[a] (mean cpm ± SD/culture (x 10^{-3}))	% Antigen (after 1.5 hr) in		
				Cells	Supernatant (TCA-insoluble)	Supernatant (TCA-soluble)
DC	POL	–	3.1 ± 0.2	49 ± 13	31 ± 5	10 ± 1
		+	2.5 ± 0.3	46 ± 3	41 ± 18	9 ± 2
	MON	–	3.3 ± 0.8	40 ± 2	35 ± 16	10 ± 2
		+	2.6 ± 0.2	48 ± 5	28 ± 5	7 ± 1
MØ	POL	–	3.9 ± 0.9	45 ± 7	28 ± 4	13 ± 3
		+	4.0 ± 0.5	53 ± 9	25 ± 6	9 ± 2
	MON	–	3.2 ± 0.5	46 ± 4	21 ± 4	12 ± 1
		+	3.1 ± 0.3	51 ± 3	23 ± 5	9 ± 1

[a] Quadruplicate cultures (1.7 x 10^6 cells/0.5 ml/culture) were incubated with 50 μg/ml POL or MON (65,000 cpm/μg) ± 0.3 mM chloroquine for 2 hr. After washing and estimation of radioactivity, the cells were incubated for a further 1.5 hr, and the amounts of antigen remaining in cells and supernatant fluids (soluble or insoluble in 10% TCA) were measured. The results are expressed as a percentage of the amount initially cell-bound (column 4).

TABLE 26

Presence of Soluble Antigen in BCG Filtrate

Antigen[a] Dilution	Proliferative Response[b] (mean cpm ± SD/culture (x 10^{-3}))	
	BCG	BCG filtrate[c]
1/10	104 ± 3	64 ± 3
1/20	107 ± 7	45 ± 8
1/40	91 ± 3	24 ± 1
1/80	80 ± 4	14 ± 1
1/160	87 ± 4	6
1/320	57 ± 6	3

[a] BCG (7 mg/ml) or BCG filtrate were included in culture at the indicated dilutions.

[b] Responders were popliteal LN from DBA/2J mice which had been immunized in the hind footpads with CFA 8 days previously. The mean background response of LN cells without antigen was 1.2 cpm (x 10^{-3})/culture. SD < 1000 cpm are not shown.

[c] BCG organisms (7 mg/ml in saline) were removed by filtration through a 0.45 μm Millipore filter.

TABLE 27

P388AD.4 Presentation of CP and Soluble Antigens in CP Filtrate

APC		Proliferative Response[a] (mean Δcpm ± SD/culture (x 10^{-3}))[b]	
Type	No./Culture (x 10^{-3})	CP[b]	CP filtrate[b]
—	0	0.1	0.1
Spleen Cells[c]	74	2.2 ± 0.4	ND[e]
	220	10.8 ± 0.8	4.6 ± 0.1
	670	43.7 ± 7.9	16.5 ± 3.0
	2000	41.5 ± 1.3	28.2 ± 5.3
P388AD.4[d]	3	< 0.1	3.3 ± 0.5
	10	< 0.1	8.2 ± 1.4
	30	< 0.1	12.1 ± 1.5
	100	2.3 ± 0.8	< 0.1

[a] The proliferative response of a DBA/2J T cell line specific for CP was assessed. Responses to APC without antigen have been subtracted. SD < 1000 cpm are not shown.

[b] CP was included in culture at 10 μg/ml. To prepare CP filtrate, 1 ml CP in PBS (7 mg/ml) was spun in an Eppendorf centrifuge for 10 min. The supernatant was removed, diluted to 15 ml with RPMI-10% FBS, filtered through a Millipore 0.45 μm filter, and 0.1 ml/culture was added (final volume 0.3 ml/culture). This concentration gave optimal proliferative responses.

[c] DBA/2J, irradiated (2000 rad).

[d] Mitomycin C treated.

[e] ND, not determined.

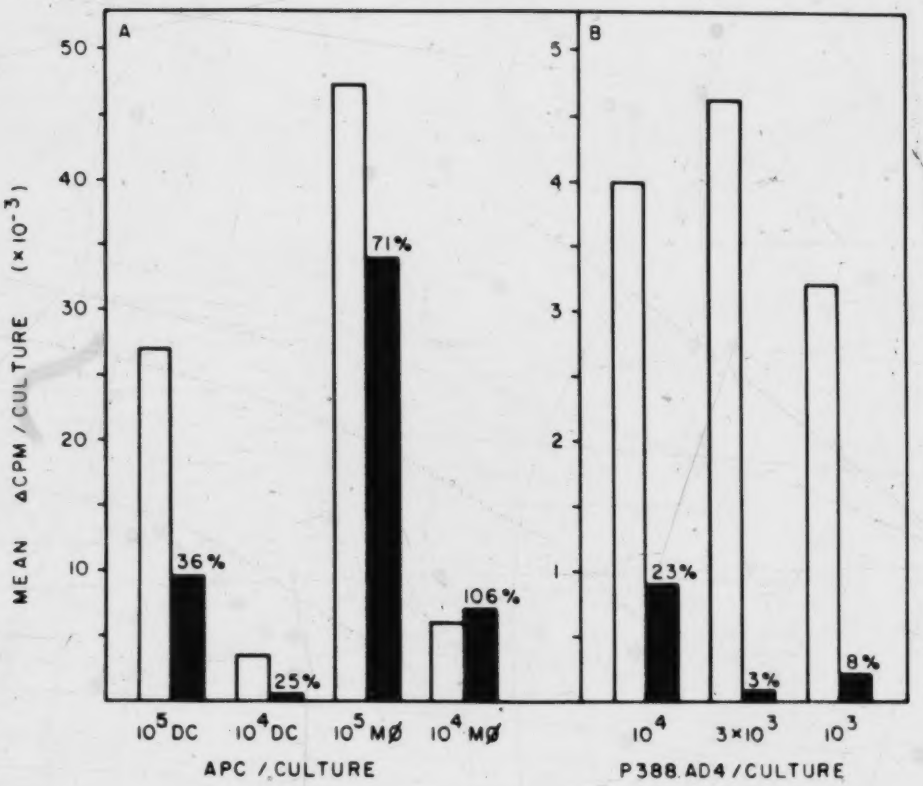

Figure 18. Presentation of glutaraldehyde-fixed CP by splenic MØ, DC, and P388AD.4. The proliferative responses induced by CBA/CaJ splenic MØ or DC (A) or P388AD.4 (B) presenting normal (open bars) or glutaraldehyde-fixed (solid bars) CP (3.5 μg/culture) to syngeneic CP-specific T cell lines were assessed. Values shown above the hatched bars represent the proliferative response induced by glutaraldehyde-fixed CP expressed as a percentage of the proliferative response induced by normal CP. Standard deviations were < 10% of the mean.

supply processed antigen. However, this selective reduction of immunogenicity after fixation was not fully correlated with reduction in antigen shedding, since the release of soluble radioactivity from ^{125}I-labeled CP in 2 hr at 37° C was reduced by only 45% (from 32,440 ± 1,738 to 18,252 ± 370 cpm).

G. Synergy Between MØ and DC for Presentation of Soluble and Particulate Antigens

The preceeding experiments suggest that pure populations of nonphagocytic cells can present whole bacterial antigens, and this is aided, at least in part, by antigen shedding from the organisms. However, the functional dichotomy between MØ and DC indicates that cell cooperation may be involved in the presentation of large soluble or particulate antigens. Thus, MØ may degrade antigens into smaller fragments which can then be pinocytosed and presented by DC. To test this possibility, mixtures of splenic MØ and DC were compared with splenic MØ or DC alone for the activation of CP- or KLH-specific T cell lines. Syngerism between the two cell types was clearly observed for the presentation of both antigens (Figure 19). Thus, the T cell responses obtained with the APC mixtures were greater than the sums of the responses induced by either type separately. Synergy was also observed for mixtures of P388AD.4 and the MØ-like cell line P388D$_1$ (Koren *et al*, 1975) (Figure 20), both of which were derived from the P388 leukemia. P388D$_1$ cells are Ia$^-$ (confirmed by FACS analysis (not shown)) and devoid of antigen presenting activity but their admixture with small numbers of P388AD.4 cells greatly augmented the proliferative response (Figure 20).

There are at least two possible contributions by the MØ to the cellular synergy described above. They could provide processed antigens or nonspecific monokines such as IL-1. The evidence obtained favors the first alternative, since neither IL-1 nor macrophage supernatants (MØ-SN) could synergize with DC (Figure 21). The concentration of recombinant IL-1 used induced maximal thymocyte proliferation (Table 28) and restored the ability of APC treated with glutaraldehyde or UV irradiation to stimulate KLH-specific T cells (Table 29). Furthermore, pretreatment of the MØ with chloroquine (0.3mM for 2 hr) abrogated their ability to synergize with DC (Figure 21). Finally, supernatants from

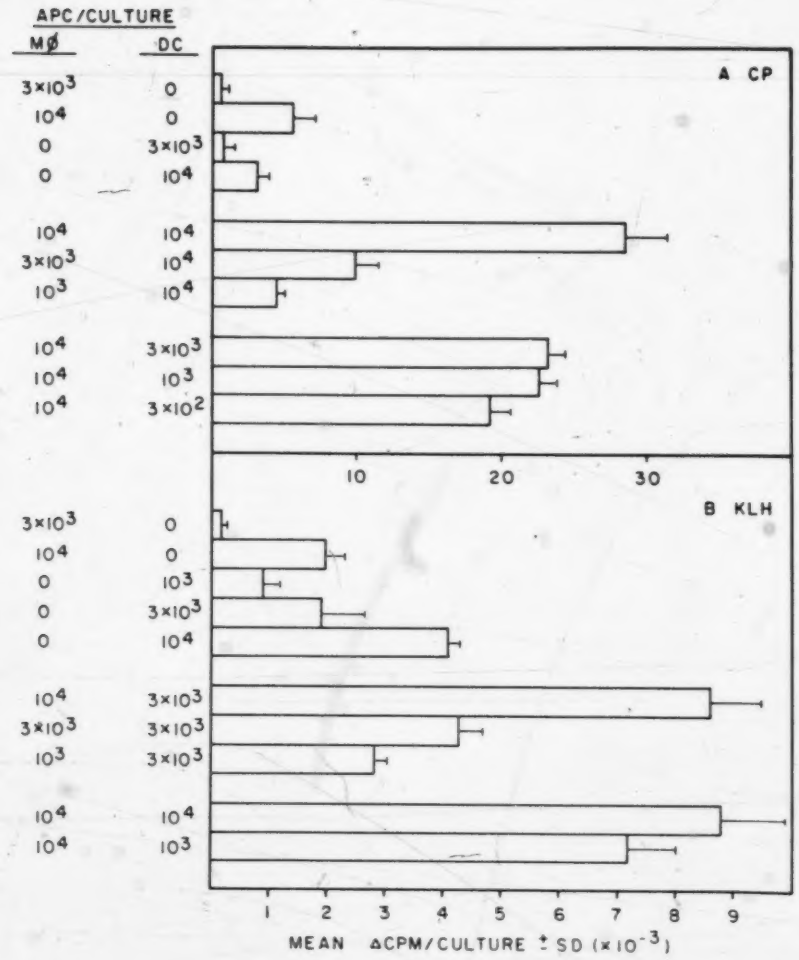

Figure 19. Synergy of splenic MØ and DC for antigen presentation. Results represent the proliferative response of a CBA/CaJ CP-specific (A) or KLH-specific (B) T cell line to CP (3.5 μg/culture) or KLH (15 μg/culture), respectively, presented by the mixtures of CBA/CaJ splenic MØ and DC indicated. The responses to APC alone have been subtracted.

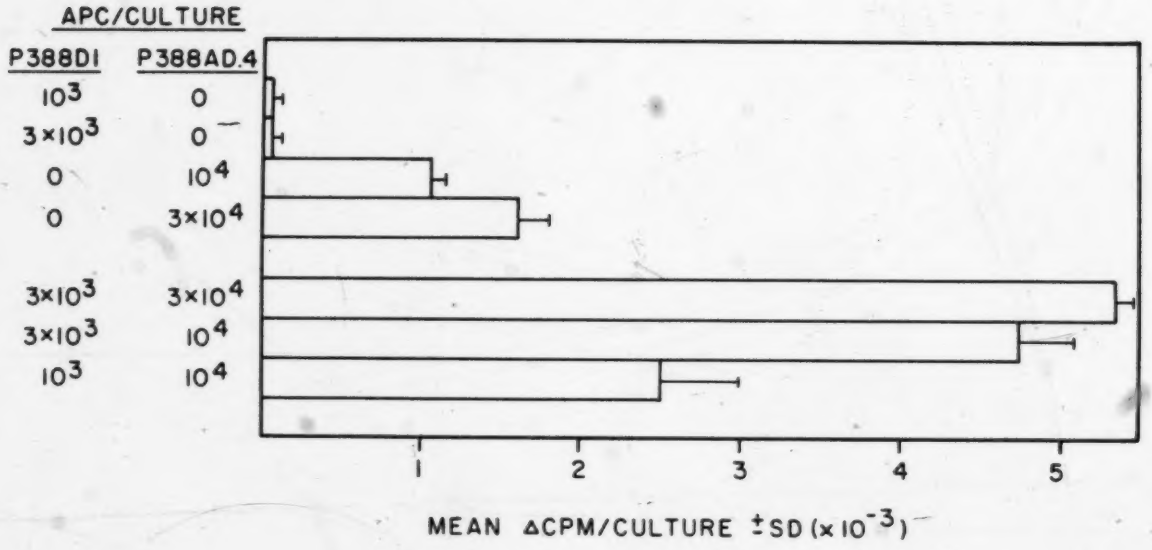

Figure 20. Synergy of MØ-like and DC-like lines for presentation of BCG. The proliferative response of a DBA/2J BCG-specific T cell line to BCG (3.5 μg/culture) presented by the indicated mixtures of $P388D_1$ and P388AD.4 (mitomycin C treated) is shown. The responses to APC alone have been subtracted.

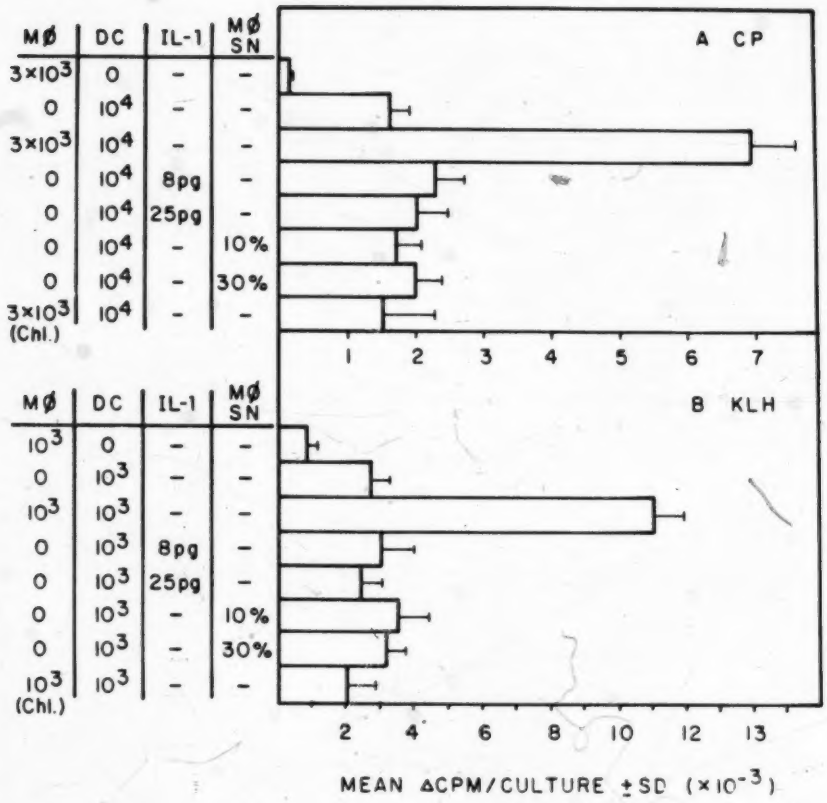

Figure 21. Chloroquine sensitivity of MØ function in MØ - DC synergy and the inability of IL-1 or MØ supernatants to replace MØ. Conditions for CP-specific (A) and KLH-specific (B) T cell proliferation were the same as those described for Figure 20, except for chloroquine (chl.) pretreatment of MØ (0.3 mM for 2 hr) where indicated. Pure recombinant murine IL-1, or supernatants from CBA/CaJ splenic MØ cultured at 10^6/ml for 3 hr were included where indicated. The concentrations of IL-1 used induced thymocyte proliferation (Table 28) and antigen-specific T cell proliferation (Table 29).

TABLE 28

Thymocyte Assay for Interleukin-1

IL-1[a] (pg/culture)	Proliferative Response[b] (mean cpm ± SD/culture)	
	IL-1 alone	IL-1 + Con A
225	86 ± 32	1,187 ± 52
75	52 ± 9	3,844 ± 525
25	61 ± 22	4,995 ± 876
8	68 ± 31	1,333 ± 210
3	39 ± 24	126 ± 59
0	58 ± 35	85 ± 37

[a] Pure recombinant murine interleukin-1 (Lomedico *et al*, 1984).

[b] Thymocytes from C3H/HeJ mice 4-8 wks old were cultured with or without Con A and with or without various concentrations of pure recombinant IL-1.

TABLE 29

IL-1 Activity in Antigen-Specific T Cell Proliferation†

APC Treatment[b]	IL-1[c] (pg/culture)	Proliferative Response[a] (mean Δcpm ± SD/culture (x 10^{-3}))
medium	-	33.5 ± 4.8
glutaraldehyde	-	2.0 ± 0.9
glutaraldehyde	25	23.0 ± 2.6
UV	-	2.8 ± 0.7
UV	25	25.7 ± 1.9

[a] CBA/CaJ T cell line specific for KLH added at 10^4/culture. Responses to unpulsed APC have been subtracted.

[b] CBA/CaJ splenic adherent cells were pulsed with KLH (100 μg/ml for 90 min at 37° C) before glutaraldeyhde fixation (0.05% for 30 s, followed by neutralization with 0.2 M lysine, Shimonkevitz *et al*, 1983) or UV irradiation (837 microwatts/cm² for 5 min, DeFreitas *et al*, 1983).

[c] The thymocyte proliferative response to IL-1 ± Con A is shown in Table 28.

† Done in collaboration with Dr. A. Sinha.

antigen-pulsed MØ, which have been reported to contain antigen-Ia complexes which can activate T cells in the absence of APC (Erb and Feldmann, 1975b; Puri and Lonai, 1980; Friedman *et al*, 1983; Puri *et al*, 1985), could not replace MØ and antigen for synergy with DC (Table 30). This suggests that MØ are required for the degradation of particulate or soluble complex antigens into smaller fragments which can then be presented by other MØ or DC for T cell activation.

TABLE 30

Failure of MØ Supernatants to Replace MØ in MØ - DC Synergy

No. MØ/Culture[a] (x 10^{-3})	Proliferative Response[b] (mean Δcpm ± SD/culture (x 10^{-3}))				
	No. DC/Culture (x 10^{-3})[a]				
	0	0.3	1	3	10
0	< 0.1	< 0.1	< 0.1	0.2	1.6
1	< 0.1	0.1	0.4 ± 0.2	1.4 ± 0.2	2.4 ± 0.5
3	1.0 ± 0.1	2.0 ± 0.9	3.5 ± 0.4	3.8 ± 0.2	7.0 ± 0.8
10	4.9 ± 0.8	6.4 ± 0.6	6.9 ± 0.1	9.2 ± 1.3	10.1 ± 1.6
Dilution MØ-SN-A[c]					
1/3		< 0.1	< 0.1	0.2	1.4 ± 0.4
1/9		< 0.1	< 0.1	0.2 ± 0.1	0.9 ± 0.1
1/27		< 0.1	< 0.1	0.2 ± 0.1	1.2 ± 0.3
1/51		< 0.1	< 0.1	0.3 ± 0.1	1.0 ± 0.1
Dilution MØ-SN-B[c]					
1/3		0.1	0.1	0.3 ± 0.1	1.0 ± 0.3
1/9		< 0.1	0.1	0.2 ± 0.1	1.3 ± 0.3
1/27		< 0.1	0.1	0.4	1.8 ± 0.1
1/51		< 0.1	< 0.1	0.2 ± 0.1	1.2 ± 0.1

[a] Splenic MØ and DC were purified from CBA/CaJ spleen cells.

[b] The proliferative responses induced in a CBA/CaJ CP-specific T cell line by the various combinations of APC (or MØ supernatants) with (MØ-SN-A) or without (MØ-SN-B) CP (7 μg/ml CP), were assessed. The responses to APC alone have been subtracted. SD < 100 cpm are not shown.

[c] Adherent splenic MØ in Falcon 100 x 20 mm tissue culture dishes (2.4 x 10^6/dish in 7 ml RPMI with 10% FBS and 2-ME) were left unpulsed, or were pulsed with 70 μg/ml (washed) CP for 90 min at 37° C. After 4 washes with warm medium, MØ were cultured for a further 6 hr. The culture supernatants from unpulsed (SN-A) and CP-pulsed (SN-B) MØ were then collected, filtered through a Millipore filter (diameter 0.45 μm), and used at the indicated dilutions.

IV. DISCUSSION

The experiments presented in this thesis demonstrate that MØ and DC possess distinctive, but overlapping, immunostimulatory activities that are consistent with their cellular characteristics. Thus, DC, which express constitutively high levels of Ia, were particularly effective at MLR stimulation, whereas MØ, possibly by virtue of their phagocytic and lysosomal activity, were better at presenting whole bacterial organisms to antigen-specific T cell lines. Both cell types were capable of presentating a variety of soluble antigens. Xenogeneic antigens, but not allogeneic or syngeneic Ia, had to be processed by a chloroquine-sensitive, presumably lysosomal, pathway to be rendered immunogenic for T cells, although differences between the reliance of MØ and DC on this pathway were discernable, especially for small antigens. The dichotomy of APC functions observed made possible the collaboration of MØ and DC for processing and presentation of complex antigens for T cell activation.

A. Syngeneic MLR

The most striking difference in the immunostimulatory functions of MØ and DC is in syngeneic MLR activation (Figures 6 and 8). DC were always the most effective stimulators. Splenic MØ were consistently less active, and this appeared to be due to true failure to stimulate rather than nonspecific suppression (Table 4). The low, but significant level of splenic MØ-induced syngeneic T cell proliferation observed here (Figures 6 and 8) and by others (Sunshine *et al*, 1982) has been ascribed to contaminating DC (Nussenzweig and Steinman, 1980), but it was also observed with small activated, BM-MØ (100% pure) (Figure 8). Thus, although DC are the most active stimulators of syngeneic MLR, MØ are not totally inactive in this regard. Although the DC preparations was only 70-80% pure, the functional differences observed between MØ and DC were too great to be accounted for by cellular impurities.

Recent work on chimeras and nude mice bearing thymus grafts has indicated that the syngeneic MLR is a manifestation of self Ia recognition by T cells (Glimcher *et al*, 1981,

1982c). The potency of DC in this regard may reflect their role in defining self Ia and hence influencing the T cell repertoire that develops during ontogeny in the thymus. However, the notion that the syngeneic MLR reflects T cell activation by self Ia in the absence of foreign antigens has been challenged (Huber *et al*, 1982; Kagan and Choi, 1983). Regardless of the true specificity of this response, DC are uniquely effective stimulators. One report suggested that this may be due to 10-20 fold greater levels of Ia (as assessed by a cellular radioimmunoassay) on DC than on splenic MØ (Nussenzweig *et al*, 1981). This conclusion is not supported by the FACS data presented in Figure 15, which demonstrates that differences in Ia expression between DC, splenic MØ and small activated BM-MØ were small. This disagreement with Nussenzweig *et al* (1981) with respect to Ia expression on splenic MØ could have been due to variation in the degree of MØ activation, or to the different assay systems employed. Nevertheless, there is agreement concerning the functional differences between MØ and DC for syngeneic MLR activation (Figures 6 and 8, Nussenzweig and Steinman, 1980). Reports that activated T cells (Mingari and Moretta, 1982), B cells (von Boehmer *et al*, 1972; Gottlieb *et al*, 1979; Katz *et al*, 1980; Shin *et al*, 1981; DeKruyff *et al*, 1985b) and other non-DC, non-MØ cells (Bienenstock *et al*, 1985) stimulate syngeneic or autologous MLR suggest that perhaps Ia$^+$ MØ are conspicuous in their ineffectiveness as stimulators for this response. The reason for this is still obscure.

B. Allogeneic MLR

Highly purified splenic MØ and absolutely pure BM-MØ were used in an effort to resolve the controversy concerning the role of MØ in allogeneic MLR stimulation. The data in Figure 6 indicate that purified splenic MØ are effective stimulators of allogeneic MLR, and are comparable to DC in this regard, in agreement with Sunshine *et al* (1982). A reduction in MLR was observed at high concentrations of both MØ and DC, and therefore was probably not the result of nonspecific suppression by MØ. Rather, it represents responses that had passed their peak by the time of the thymidine pulse. Thus, the proliferation in cultures stimulated with high concentrations of APC peaked at an earlier time-point (data not shown).

The similarity of the dose-response curves of MØ and DC suggests that the activity of the MØ fraction was not due to cross-contamination by DC. Furthermore, the splenic MØ used were very pure (> 98% FcR^+ MØ). Although it is unlikely that < 2% contaminating DC accounted for all the stimulatory activity of the MØ fraction, it is possible that MØ synergized with low numbers of contaminating DC. In this regard, Naito *et al* (1984) have recently shown that MØ enhance allogeneic MLR stimulation by suboptimal numbers of DC. Therefore, it was essential to show that a population of MØ free from DC contamination could stimulate MLR. Thus, pure culture-derived BM-MØ were compared with DC and splenic MØ for the ability to stimulate allogeneic MLR.

The inactivity of unactivated BM-MØ fractions as stimulators of MLR can be attributed to the low percentage of Ia^+ cells contained in this MØ population (Figure 15). Several other investigators have also reported that unactivated culture-derived BM-MØ contain few (< 20%) Ia^+ cells (Mottram and Miller, 1980; Calami *et al*, 1982; Lee and Wong, 1982; Warren and Vogel, 1985). In contrast, Stern *et al* (1979) found that 50% of culture-derived BM-MØ were Ia^+ without deliberate activation. However, these investigators used lung cell conditioned medium as a source of CSF-1. This conditioned medium has been shown to generate many more Ia^+ MØ than LCM (Lee and Wong, 1980), probably due to the presence of interferons. Less easily explainable is the report by Schook *et al* (1982) that BM-MØ grown under nearly identical conditions to those used here yielded 60% Ia^+ MØ. Perhaps the mice used in these studies had high endogenous interferon levels due to environmental infection, and thus MØ precursors could have been activated before culture. Indeed, on a few occasions throughout the course of my studies, unactivated BM-MØ were found to express high levels of Ia, but this was not a reproducible finding, and thus may be attributable to environmental infection.

Overnight incubation with lymphokines and LPS induced a dramatic increase in both the number of Ia^+ MØ (from 13% to 65%) and the level of Ia expressed per cell (as represented by the mean fluorescence intensity (Figure 14), although the distribution of cells with different Ia densities was very wide. Warren and Vogel (1985) similarly observed a great increase (from

< 10% to 90%) in the number of Ia$^+$ BM-MØ after culture with recombinant IFN-γ, as assessed by complement-mediated cytotoxicity and ELISA. The much more modest increase (from 2.5% to 25%) in Ia$^+$ BM-MØ observed by Calami *et al* (1982) following their Ia-induction protocol may have been an artificially low estimate due to subjective identification of Ia$^+$ cells by fluorescence microscopy. With respect to this possibility, the FACS analysis depicited in Figure 15 shows that many of the activated BM-MØ expressed low levels of Ia, which may not have been detectable visually.

Small immature BM-MØ activated to induce Ia expression were excellent stimulators of allogeneic MLR (Figure 7). Large (mature) activated BM-MØ were not stimulatory despite substantial surface Ia (Figure 15). This is most likely due to the nonspecific suppressive activity of these MØ (Lee and Wong, 1982). Similarly, the failure of Steinman *et al* (1980) to observe allogeneic MLR stimulation by activated peritoneal MØ (which were 95% Ia$^+$) can probably be attributed to the dominant suppressive activity of this MØ population (Lee and Berry, 1977). Unexpectedly, the activity of splenic MØ relative to DC (Figure 8) was somewhat lower than in the previous experiment (Figure 6), and was variable in other experiments (not shown). Although splenic MØ are mostly small (Lee, 1980b) and immature (Steinman *et al*, 1979), and should therefore not have been suppressive, it is possible that different splenic MØ preparations had varying proportions of activated suppressive MØ (possibly due to different degrees of environmental infection), thus causing some variability in the immunostimulatory capacity of unfractionated splenic MØ. Nevertheless, MØ were effective stimulators of allogeneic MLR, and, in the absence of the suppressive effects of large MØ, were nearly as effective as DC (Figure 8).

This conclusion depends heavily on the purity of the BM-MØ, which were grown in medium supplemented with LCM as a source of CSF-1. LCM supports sparse early growth of granulocytes under some conditions (Stern *et al*, 1979), but they die off by the fifth day of culture (Lee and Wong, 1980). Granulocyte growth was further minimized by seeding low numbers of BM cells (Lee and Wong, 1982). Adherent cells harvested in the middle of the exponential growth phase (day 5 or 6) are 100% FcR$^+$, esterase-positive, phagocytic, and have

the characterisitc morphology of MØ (Lee and Wong, 1980). Similar high levels of purity have been reported by other investigators using similar growth conditions (Mottram and Miller, 1980; Calami *et al*, 1982; Schook *et al*, 1982; Walker *et al*, 1985). Most importantly, growth of DC has never been observed. In contrast to MØ, conditions for the *in vitro* growth of DC from BM precursors have not been well defined. In 1982, Bowers *et al* reported success in growing rat DC from precursors present in BM and peripheral blood using supernatant from mitogen-activated spleen cells, but no additional information has since been published. More recently, Clayberger *et al* (1985) described a cloned murine DC-like line derived from limiting-dilution culture of murine spleen cells with contitioned medium from Con A-stimulated spleen cells. Although this cell line has DC-like morphology, surface markers (Ia^+, $Thy\text{-}1^-$, Ig^-, FcR^-) and is nonphagocytic, no data on its MLR-activating capacity or APC function were provided, making it difficult to draw conclusions. In fact, it may actually represent an atypical T cell, since it produces a B cell stimulatory factor similar to those produced by T cells (reviewed in Howard *et al*, 1984). Inaba *et al* (1981) also described a DC-like line that secretes a B cell mitogenic factor. However, the isolation of DC-like lines by both of these groups was serendipitous and has not been repeated.

In summary, these studies have provided strong evidence that at least some MØ subpopulations are effective stimulators for allogeneic MLR. The past controversy over this matter can probably be attributed to MØ heterogeneity and varying numbers of suppressive activated MØ. The finding that both MØ and DC were effective stimulators of allogeneic MLR suggests that the "passenger leukocytes" that are responsible for the rejection of some types of allograft (Lafferty *et al*, 1983) may consist of both cell types. Thus, strategies for depletion of passenger leukocytes from donor organs should be designed accordingly.

C. Antigen Presentation

DC and MØ were about equal in the ability to present soluble antigens, although DC were more effective at high cell concentrations, possibly due to the suppressive activity of MØ at these concentrations. Other workers have shown that DC and some nonphagocytic B cell

and DC-like lines can bind and present protein antigens such as OVA, turkey γ-globulin, or KLH (Chestnut *et al*, 1982a; Cohen and Kaplan, 1983; Miyazaki and Osawa, 1983; Sunshine *et al*, 1983). Thus, the minimal endocytic and catabolic activities of these cells are sufficient for producing immunogenic moieties from soluble protein antigens.

In contrast to soluble antigen presentation, splenic MØ were superior to DC at presenting whole bacterial organisms, presumably because their antigen-handling machinery is better suited for the uptake and processing of particulate antigens. Small activated BM-MØ (fraction A) were about three times as efficient (on a per cell basis) as splenic MØ and were the most active APC in this system (Figure 12). This is consistent with a role for the increased phagocytic and degradative functions of activated BM-MØ in processing particulate antigens. Contrary to this, Kaye *et al* (1985) reported that splenic DC were the most effective presenters of whole BCG organisms when compared to splenic MØ and peritoneal MØ. It should be noted that the peritoneal MØ used in this study were not activated and contained only 2-10% Ia$^+$ cells. Furthermore, the splenic MØ population used was nonadherent after overnight incubation and could represent a distinct MØ subset. In my experience, many splenic MØ remain adherent after overnight culture and must be dislodged by vigorous pipeting (unpublished observations). It is also possible that the DC were presenting soluble antigen shed from the BCG (see below).

Although only a small proportion of antigen ingested and degraded by APC can be expected to be participate in immune induction (Unanue, 1981,1984), the possibility that differences in antigen uptake could have accounted for the functional differences observed among APC subsets was examined. Clearly, large BM-MØ, which functioned poorly as APC, were not deficient in the uptake of particulate or soluble antigens (Table 17). Moreover, the smallest BM-MØ bound approximately 50% less CP than the other BM-MØ fractions but were the most efficient APC for this antigen, indicating that the gross amount of antigen taken up by a cell does not correlate with its efficiency as an APC. This implies that antigen uptake was not limiting in this system. For other APC, such as resting B cells, poor antigen uptake probably is related to the inefficent APC function of these cells (Chestnut *et al*, 1982b). No difference between MØ and DC was observed for the uptake of particulate and soluble antigens

(Tables 18 and 24). In contrast, P388AD.4 cells were selectively deficient in CP uptake (Table 17). This cell line has been reported to take up soluble antigens poorly (Cohen and Kaplan, 1983); this would appear to disagree with the results reported here for the uptake of KLH by P388AD.4 (Table 17). However, Cohen and Kaplan used turkey γ-globulin, which is probably taken up much more efficiently by FcR^+ than FcR^- APC (Heusser *et al*, 1977), whereas FcR^+ and FcR^- APC should take up comparable amounts of a non-γ-globulin antigen such as KLH. The results in Table 17 are consistent with this idea.

In view of the relative inefficiency of P388AD.4 to take up CP, the failure to observe a similar selective deficiency in the uptake of particulate antigens by DC is surprising. The most likely explanation lies in the different protocols used for studies of antigen uptake by DC and P388AD.4. Splenic DC are nonadherent after overnight culture and fail to re-adhere once detached from the culture dish (Steinman and Nussenzweig, 1980). This characteristic necessitated that DC and splenic MØ be pulsed with CP while free in suspension. Since CP was removed by centrifugation, some bacteria could have been pelleted with the cells, thus accounting for the failure to observe a difference in particulate antigen uptake between DC and MØ. Since the DC-like and MØ-like lines used in these studies grew as adherent monolayers, the antigen-pulse was performed *in situ*, such that bacteria could be easily removed by washing (see *Materials and Methods*).

Regardless of the potential differences in the uptake of particulate antigens by MØ and DC or P388AD.4 during a 2 hr pulse period, the differences in function between the different APC types were observed even when antigen was present throughout the culture period (Figure 12 and Table 14). This reinforces the notion that antigen availability was probably not a limiting factor in this system. The antigen uptake studies with P388AD.4 indicate, however, that nonphagocytic APC may be selectively deficient in the uptake of particulate antigens, such as whole bacteria. In addition, the poor degradative capacity of P388AD.4 for CP (Table 19) may be germane to its inefficiency in presenting that antigen.

Quantitative variation in Ia expression has been reported to directly influence APC function (Matis *et al*, 1983; Zlotnik *et al*, 1983; Lechler *et al*, 1985). Therefore, the poor

activity of large BM-MØ as APC for presentation of soluble and particulate antigens (and MLR activation) may be due to lower levels of surface Ia, compared to DC, splenic MØ and small activated BM-MØ (Figure 15). It should be noted, however, that MØ, especially large mature MØ, exhibit marked autofluorescence (Walker *et al*, 1985). Thus, the proportion of Ia$^+$ MØ detected by FACS analysis may be an underestimate. Consistent with this, large activated BM-MØ expressed similar or greater levels of Ia as compared to small BM-MØ when assessed by an ELISA technique (not shown). Furthermore, the level of Ia on APC is not always positively correlated with effectiveness of antigen presentation. For example, Walker *et al* (1982b) found that some B cell tumors with barely detectable levels of Ia presented antigen to T cell hybridomas. Similarly, Glimcher *et al* (1982b) found that the inability of some Ia$^+$ B cell tumors to present antigen or stimulate MLR was not correlated with Ia antigen density. Finally, Geppert and Lipsky (1985) reported a differential ability of endothelial cells and fibroblasts to function as APC despite comparable Ia density. Thus, although a certain threshold density of Ia is probably required to activate T cells (Lechler *et al*, 1985), increasing Ia density beyond this threshold may not greatly enhance APC function. Differences in APC function between cell types can probably be attributed to other cellular characteristics. In the case of large activated BM-MØ, suppressive activity probably masks any APC function that these cells might possess (Lee and Wong, 1982).

The ability of DC to present particulate antigens was unexpected in view of the low endocytic capacity of this cell type (reviewed in Steinman, 1981). The fact that P388AD.4 also presented CP suggests that nonphagocytic APC can process and present whole bacterial antigens, albeit inefficiently, in the absence of the potential for MØ contamination. The manner by which the bacterial antigens are processed is probably critical, especially for DC and P388AD.4 which are nonphagocytic and have low degradative activity. Kaye *et al* (1985) suggested that DC could perform the processing at the cell membrane, but there is no evidence for this. Release of soluble antigen by the bacteria (to be pinocytosed by DC) is more likely, and evidence that this can occur was presented (Tables 25 and 26, Figure 18). However, the inhibition of antigen release by glutaraldehyde treatment was incomplete (45%), and cannot

fully explain the much larger decrease in CP immunogenicity after fixation (Figure 18). This suggests that in addition to a dependence on shed antigen, the nonphagocytic APC may also have a reduced capacity to process fixed bacteria. The fact that glutaraldehyde-fixation of CP produced a more profound reduction of antigen-presenting activity in P388AD.4 than in DC (Figure 18) indicates that the DC preparation could have contained a few contaminating MØ to help in antigen processing.

D. Antigen Processing

Using chloroquine as a lysosomal inhibitor (de Duve *et al*, 1974), it was shown that antigen processing by splenic MØ and DC requires chloroauine-sensitive (presumably lysosomal) functions (Tables 20-22), in agreement with other studies with peritoneal MØ, B cell tumors, and unfractionated splenic APC (Chestnut *et al*, 1982a; Lee *et al*, 1982; Ziegler and Unanue, 1982). Consistent with other reports (Grey *et al*, 1982; Ziegler and Unanue, 1982) chloroquine had no effect on antigen uptake (Tables 18 and 24). The inhibitory effect of chloroquine on antigen catabolism by mature peritoneal MØ (Grey *et al*, 1982; Ziegler and Unanue, 1982) and BM-MØ (Lee *et al*, 1982) has been clearly demonstrated. However, MØ from spleen are small (Lee, 1980b) and immature (Steinman *et al*, 1979), and it is not surprising that antigen degradation into acid-soluble material (in 1.5 hr) was marginal and the effect of chloroquine was correspondingly small (Table 24). Nevertheless, the sensitivity of antigen-presenting function to chloroquine suggests that antigen processing did take place, even though the amount of processed material responsible for T cell stimulation might have been very small. Grey *et al* (1982) obtained similar results with antigen-presenting B cell tumors.

Inhibitor studies are always subject to the criticism that the inhibitor could have additional, undetected effects on cell physiology other than the one it is noted for. Indeed, chloroquine has recently been shown to inhibit the biosynthesis of whole cellular Ia (Nowell and Quaranta, 1985), and this could explain its inhibitory effect on antigen presentation. However, since the chloroquine sensitivity of the response varied with the type of antigen, inhibition of Ia expression was probably not the dominant effect of chloroquine in these

studies. Furthermore, it took several hours for the block in Ia synthesis to be detected, thus, the Ia-dependent part of the T cell activation process could have already been completed.

The specificity of the chloroquine effect was highly concentration dependent, even though APC viability was not affected after treatment. The optimal chloroquine concentration for selective abrogation of the presentation of large complex antigens showed some variation in different experiments. This could have been due to varying degrees of environmental infection and hence MØ activation in the animals. Although MLR stimulation by purified DC and MØ was not entirely unaffected by chloroquine in some experiments, it was always less sensitive to chloroquine than the presentation of complex xenogeneic antigens (Table 22). This observation has been confirmed in the human system (Scala and Oppenheim, 1983). Furthermore, Finnegan *et al* (1985) showed that a T cell clone with dual specifity for self MHC plus antigen and allogeneic MHC had distinct processing requirements, depending on the nature of the antigen. Only the response to self MHC plus antigen was significantly inhibited by pretreatment of APC with chloroquine. Finally, experiments demonstrating effective T cell stimulation by alloantigen presented in planar membranes (Brian and McConnell, 1984) or liposomes (Weinberger *et al*, 1985) suggest that histocompatibility molecules do not need to be processed.

The processing requirement for xenogeneic antigens has been shown to vary according to the size (Lee *et al*, 1982; Buus and Werdelin, 1986c) or conformation (Büchmuller and Corradin, 1982; Streicher *et al*, 1984; Boyer *et al*, 1986) of the antigen. Here, I have presented evidence that the type of APC may also influence the processing requirement, but this also depends on the nature of the antigen being presented. For example, presentation of BCG by both DC and MØ was largely inhibited by chloroquine, whereas presentation of PPD was much more chloroquine sensitive with MØ as APC (Figure 17). Thus, for a smaller antigen like PPD, either processing is not stringently required (Lee *et al*, 1982) when presented on DC, or some processing takes place *via* a lysosome-independent (and chloroquine-insensitive) pathway. The production of immunogenic moieties by MØ appears to depend entirely on the chloroquine-sensitive pathway, regardless of the antigen.

E. MØ – DC Collaboration

The dichotomy of APC functions in T proliferative responses raises the possibility of accessory cell collaboration, with MØ and DC being primarily responsible for antigen catabolism and antigen presentation, respectively. To explore this possibility, limiting numbers of MØ and DC (and also P388D$_1$ and P388AD.4) were mixed in various proportions, and good synergistic effects were observed. In this system, it appeared that MØ were limiting. Thus, a 3-fold reduction in the number of MØ co-cultured with 10^4 DC greatly reduced the CP-specific proliferative response, whereas a 30-fold reduction in the number of DC co-cultured with 10^4 MØ only marginally decreased the response (Figure 19A). Similar results were obtained for the activation of a KLH-specific T cell line (Figure 19B), and a BCG-specific T cell line (Figure 20).

MØ-DC synergy has been reported for a T-dependent B cell response (Inaba *et al*, 1981) and a T proliferative response to KLH (Miyazaki and Osawa, 1983). In both cases, MØ could be replaced by nonspecific supernatant factors. On the contrary, neither recombinant IL-1 nor MØ-SN could replace MØ in the synergistic responses observed here, and the sensitivity of MØ to pretreatment with chloroquine suggests that processing of antigen was required (Figure 21). Although the data are consistent with the transfer of fragmented antigen from MØ to DC, demonstration of supra-immunogenic antigenic moieties in the culture fluid of antigen-fed MØ was not possible (Table 29). This could have been due to the low concentration of antigen in such culture fluids. The proximity of MØ and DC in the white pulp of the spleen (Steinman *et al*, 1975), or *in vitro*, may ensure effective transfer of processed antigen to DC, which could present it directly, or after further processing. In an analogous system of APC collaboration, Roska and Lipsky (1985) recently reported that the ability of paraformaldehyde-fixed, Ia$^+$ MØ to stimulate antigen-specific T cell proliferation in the presence of soluble antigen was restored by the addition of Ia$^-$ endothelial cells or fibroblasts, which were themselves nonfunctional as APC. These results are consistent with the transfer of processed antigen from Ia$^-$ cells to Ia$^+$ cells, although antigen processing by the Ia$^-$ cells was not directly demonstrated in this study.

In summary, the data presented in this thesis show that APC function can be performed by cell types which have distinct cell-surface phenotypes and functional capabilities. A rationale for the functional dichotomy between MØ and DC was provided by the demonstration of antigen-dependent differences in efficiency of APC function. Some MØ subsets can stimulate MLR or antigen-specific T cell proliferation, depending on Ia expression and a minimum of suppressive activity. These parameters are variable and can be affected by the stage of differentiation (Lee and Wong, 1982) or activation (Adams and Hamilton, 1984) of the MØ. In contrast, the potent T cell stimulatory capacity of DC is probably due to high constitutive levels of Ia (Steinman, 1981) and lack of suppressive capabilities. MØ can enhance DC-mediated T cell activation by providing processed antigen or inhibit it nonspecifically (Lee and Wong, 1982). APC-dependent selection of T cell functions may be a further consequence of accessory cell heterogeneity (Ramila and Erb, 1983; Ramila *et al*, 1985). For example, MØ, but not DC, were reported capable of stimulating suppressor T cells (Usui *et al*, 1984). Thus, the existence of functionally diverse APC types provides an opportunity for immunoregulation at the earliest stage of immune induction.

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